



December 14, 1995

Dockets Management Branch (HFA-305)
Food and Drug Administration
Room #1-23
12420 Parklawn Drive
Rockville, MD 20857

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**re: Topical Antimicrobial Drug Products for Over-the-Counter Human Use;
Tentative Final Monograph for Health-Care Antiseptic Drug Products
Data Submission**

Dear Sir or Madam:

On behalf of Lonza Inc., I am submitting three (3) copies of the following documents in response to the Food and Drug Administration's (FDAs) Tentative Final Monograph for Health-Care Antiseptic Drug Products ("TFM") published in the *Federal Register* on June 17, 1994 (59 FR 31402).

- Volume 1 of 4
Expert Panel Review of Benzethonium Chloride: Panel Commentary
- Volume 2 of 4
Expert Panel Review of Benzethonium Chloride: Reference Documents
- Volume 3 of 4
Developmental Toxicity Study in Rats with Hyamine 1622 (Benzethonium Chloride)
- Volume 4 of 4
National Toxicology Program (NTP) Studies with Benzethonium Chloride

If you have questions regarding this submission, please contact me at (301) 652-5495.

Sincerely,

Eliot I. Harrison
Consultant to Lonza

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**EXPERT PANEL REVIEW OF
BENZETHONIUM CHLORIDE**

Volume I: Panel Commentary

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Joseph F. Borzelleca, Ph.D.
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Panel Coordinator

Mr. Eliot Harrison

December 13, 1995

Expert Panel Review of Benzethonium Chloride

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Expert Panel Review of Benzethonium Chloride

I. Introduction

An expert panel review of the existing toxicology database on benzethonium chloride (BZC) was performed for the purpose of providing Lonza Inc. with an opinion regarding the safety of using this chemical as an antibacterial agent in consumer hand soaps. The members of this expert panel were Herbert Blumenthal, Ph.D. (former Director of the Division of Toxicology, FDA Center for Food Safety and Applied Nutrition); Joseph F. Borzelleca Ph.D. (Professor of Pharmacology and Toxicology, Virginia Commonwealth University); and Gerald P. Schoenig, Ph.D. (President, Toxicology/Regulatory Services). Mr. Eliot Harrison (Delta Analytical Corporation) served as Panel Coordinator.

Prior to the panel meeting, which took place on September 6 and 7, 1995, the panel members were provided with materials to review. Additional materials were provided during and after the panel meeting. It is believed that all of the pertinent information which currently is available on BZC was provided for review and consideration by the panel members.

In addition to what is believed to be all of the available data on BZC, summarized data from two recent comprehensive data development programs conducted with two other quaternary ammonium compounds [alkyldimethylbenzylammonium chloride (ADBAC) and didecyldimethylammonium chloride (DDAC)] were provided for review. Also provided were chemical structures and comparative toxicity data on BZC, ADBAC and DDAC. These data and information have been placed behind Tabs 23, 24 and 25, respectively, in the Reference Volume which accompanies this report. Another document which the panel found to be useful was a review article entitled "Final Report on the Safety Assessment of Benzethonium Chloride And Methylbenzethonium Chloride". A complete copy of this article has been placed behind Tab 15 in the Reference Volume.

The endpoints of carcinogenicity, mutagenicity, teratogenicity, and reproductive toxicity were evaluated from a qualitative perspective. Included in these evaluations were both an assessment of the adequacy of the data and an assessment of the potential for BZC to produce these types of effects. The data for systemic toxicity were evaluated from both qualitative and quantitative perspectives. For systemic toxicity, the qualitative evaluation involved an assessment of the adequacy of the data and an identification of the types of systemic effects that were observed. Available pharmacokinetic data were incorporated into this review. The quantitative evaluations for systemic toxicity involved the

determination of what the panel felt was the most appropriate no observed effect level (NOEL) for systemic effects in the animal toxicity studies conducted with BZC. Available data on eye and skin irritation and skin sensitization were reviewed from the perspective of determining if the use of BZC in consumer hand soap posed any potential problems in these areas.

This document has been prepared in two volumes. The first volume contains the Expert Panel's commentary, conclusions and recommendations. The second volume (Reference Volume) contains summaries of the larger reports and copies of the other reference materials which were available for the panel to review.

II. Review of BZC Data for Carcinogenicity, Mutagenicity, Teratogenicity and Reproductive Effects

A. Carcinogenicity and Mutagenicity

1. Data Reviewed and General Commentary

a. Carcinogenicity

Draft reports for rat and mouse dermal carcinogenicity studies, which were conducted under the auspices of the National Toxicology Program (NTP), were provided to members of the panel for review. Detailed summaries of these studies were prepared and are provided in the Reference Volume behind Tab 1.

Each of these studies consisted of three treatment groups and a solvent control group. Sixty rats or mice of each sex were included in each group. The duration of dose administration was two years. Dermal doses of 0.15, 0.5 and 1.5 mg/kg/day were evaluated in both studies. In all cases the dosing solutions were prepared using 95% ethanol (USP Grade) and were applied five days per week. These studies were considered to be well conducted and no evidence of carcinogenicity was observed in either study. The panel felt that the one limitation of these studies was that dermal irritation and dose volume limited the actual doses of BZC which could be administered to rather low dose levels.

The panel also considered the following additional carcinogenicity studies:

- An eighteen-week study in which male mice received subcutaneous injections of minced tissue collected from injection sites previously injected with BCZ (Reference Volume, Tab 2);
- A seven-month study in which a sensitive strain of female mice received single or multiple intravenous injections of BZC followed by an examination of lung tissues (Reference Volume, Tab 2);
- A 23- to 29-week cocarcinogenicity study in which male mice received subcutaneous injections of BZC (Reference Volume, Tab 2);
- A fifteen-month carcinogenicity study in which male and female mice received single subcutaneous injections of BZC (Reference Volume, Tab 3);
- An eighteen-month study in which male and female mice were given multiple (15 to 16) subcutaneous or intraperitoneal injections of BZC over a 30- to 32-week period (Reference Volume, Tab 4);
- An eighteen-month study in which male and female rats received subcutaneous injections of BZC twice weekly for 52 weeks followed by a six-month observation period prior to sacrifice (Reference Volume, Tab 5);
- A two-year study in which rats were fed BZC in the diet (Reference Volume, Tab 6).

With the exception of the eighteen-month study in which rats received subcutaneous injections of BZC (Reference Volume, Tab 6), no evidence of carcinogenicity was observed in any of these studies. In the 18-month rat study, an increase in nonmetastisizing fibrosarcomas was observed only at the sites of BZC injection. The committee noted that there is a considerable amount of literature concerning such fibrosarcomas which refers to their occurrence as a consequence of chronic local irritation and,

thus, they are of no concern in the consideration of the safe use of BZC in bacterial soaps or other topical preparations.

Summarized results of carcinogenicity studies conducted with ADBAC and DDAC by the oral route of administration at maximum tolerated doses also were reviewed and discussed. No evidence of carcinogenicity was observed in any of these studies.

b. Mutagenicity

A draft report for three mutagenicity studies conducted with BZC as part of the NTP program was available for review (Reference Volume, Tab 7). The studies included assays for reverse mutation in *S. Typhimurium* (Ames Test), sister chromatid exchange and chromosomal aberration assays in Chinese hamster ovary cells. Mutagenicity studies conducted with ADBAC (CHO/HGPRT point mutation assay and UDS in rat primary hepatocytes) and DDAC (Ames test, CHO chromosomal aberration assay, CHO/HGPRT point mutation assay and UDS in rat primary hepatocytes) also were discussed. No evidence of mutagenicity was observed in any of these studies.

2. Conclusion of the Panel Regarding Carcinogenicity and Mutagenicity

The panel members concluded that the database was adequate and that BZC did not represent a carcinogenic or mutagenic risk if used as an antimicrobial agent in consumer hand soap.

B. Teratogenicity

1. Data Reviewed and General Commentary

Detailed summaries for three rat studies and one rabbit study conducted by the Colgate-Palmolive Company in the early 1970s (Reference Volume, Tab 8), and a recent state-of-the-art rat teratology study and associated dose range-finding study conducted by Lonza Inc. (Reference Volume, Tab 9) were available for review.

Reported findings in the three earlier Colgate-Palmolive rat studies provided conflicting evidence regarding the teratogenic and/or fetotoxic potential of BZC. In two of these studies, BZC was administered from gestation days 6 through 15 and in the other BZC was administered from

gestation day 15 through lactation day 20. In these studies BZC was administered by gavage at dose levels ranging from 0.059 to 35.6 mg/kg/day.

Dams treated at levels of 35.5 mg/kg/day on gestation days 6 through 15 had a decreased weight gain. This was reflected in an increased runtiness of litters. There was also an increase in delayed ossification at this dose. Other effects were sporadic or occurred at incidences no greater than historic controls. In dams treated from gestation day 15 through lactation day 20, no effects were noted for the dams although, during the lactation phase, pup viability was decreased at the two highest dose levels. The overall no-effect level in these studies was 1.13 mg/kg/day.

In the Colgate-Palmolive rabbit study, with the exception of the high-dose group (35.6 mg/kg/day) in which 16/27 does died and marked maternal body weight depressions were observed, no evidence of teratogenicity or fetal toxicity was observed. In this group a decrease in the number of viable fetuses, an increase in the number of resorptions and a decrease in fetal body weights were observed. These findings were considered by the panel to be secondary to the marked maternal toxicity which occurred at this dose level.

In the recent state-of-the-art rat teratology study conducted by Lonza Inc., BZC was administered by gavage on gestation days 6 through 15 at dose levels of 10, 30, 100 and 170 mg/kg/day. In this study no evidence of developmental toxicity was observed even though marked maternal toxicity (clinical signs including death in 4/24 dams plus persistent decreases in body weight and food consumption) were observed at the highest dose level.

No developmental toxicity was observed in the rat and rabbit teratology studies conducted with ADBAC and DDAC.

2. Conclusion of the Panel Regarding Teratology

Because of the age of the Colgate-Palmolive studies and the conflicting results which were obtained in them, the panel decided to rely upon the recent state-of-the-art studies conducted on BZC, ADBAC and DDAC for its opinion regarding the potential of BZC to produce teratogenic or fetotoxic effects. It was noted that, in the more recent rat teratology study conducted by Lonza Inc., the animals were treated at levels up to four and one-half times those in the Colgate-Palmolive Co. studies with no fetotoxic outcomes, even though the highest treatment level, 170

mg/kg/day, resulted in marked maternal toxicity, including death, in the case of four of the dams. This suggested to the committee that the Colgate-Palmolive Co. studies should be essentially discounted and that, based upon the weight of the evidence, BZC was not considered to represent a risk for developmental toxicity if used as an antibacterial agent in consumer hand soaps.

C. Reproductive Toxicity

1. Data Reviewed and General Commentary

No studies in which BZC was evaluated for reproductive effects were located. However, the results of the two state-of-the-art two generation rat reproduction studies conducted on ADBAC and DDAC were considered by the panel. No evidence of reproductive effects was observed in these studies which were conducted at dose levels high enough to produce maternal toxicity.

2. Conclusion of the Panel Regarding Reproductive Effects

While the panel would have preferred to have reproductive toxicity data on BZC to review, it concluded that the high quality data which were available on two other quaternary ammonium compounds plus the lack of activity of BZC in the mutagenicity and teratology studies provided adequate evidence to support the position that it is very unlikely that BZC would represent a risk for reproductive effects if used as an antibacterial agent in consumer hand soaps.

D. Systemic Toxicity

1. Data Reviewed and General Commentary

Draft reports for 16-day, 13-week and two-year dermal toxicity studies conducted in the rat and mouse as part of the NTP were provided to the panel members for review. Summaries of these rat and mouse studies are provided in the Reference Volume behind Tabs 10 and 11, respectively.

In the rat studies, dose levels ranged from 6.3 to 100 mg/kg/day in the 16-day study, 1.56 to 25 mg/kg/day in the 13-week study and 0.15 to 1.5 mg/kg/day in the two-year study. In the 16-day study, decreased body weight, possibly associated with marked skin irritation, was observed at dose levels of 50 and 100 mg/kg/day. In the 13-week study, possible

treatment related effects on body weights (decreased), thymus weights (decreased), kidney weights (increased) and hypercellularity of the bone marrow were observed in animals at the high-dose level of 25 mg/kg/day. Marked skin irritation also was observed in animals at this dose level. Because of the possible association between the skin irritation and body weight effects, the small differences in organ weights when compared to control and the lack of microscopic confirmation of changes in the thymus and kidneys and the mild nature of the bone marrow findings, the panel did not feel that any of these findings represented clear evidence of systemic toxicity in any of these studies.

The dose levels in the three mouse studies were the same as those employed in the rat studies. With the exception of a slight decrease in body weight at the 25 mg/kg/day dose level in the thirteen-week study, which probably was secondary to marked skin irritation, there was no evidence of systemic toxicity observed in any of these studies.

The panel also reviewed the following studies in which systemic toxicity was evaluated:

- A four-week study in which rabbits were dermally administered two mls of a 0.1% solution of BZC five days per week; No systemic effects were noted (Reference Volume, Tab 12).
- A one-year study in which adult mongrel dogs were administered diets containing 5, 100 or 500 ppm BZC (\cong 0.125, 2.5 and 12.5 mg/kg/day, respectively); No systemic effects were noted (Reference Volume, Tab 6).
- A two-year study in which male and female rats (10/sex/group) were administered diets containing 50, 200, 1000, 2500 or 5000 ppm BZC (\cong 2.5, 10, 50, 125 or 250 mg/kg/day, respectively); Body weights were decreased and mortality was increased for animals at the 5000 ppm treatment level. Distended ceca was observed at necropsy and thinning of the cecal wall was observed microscopically in animals at the 1000, 2500 and 5000 ppm treatment levels (Reference Volume, Tab 6).

Summarized data for subchronic and chronic oral toxicity studies conducted with ADBAC and DDAC also were reviewed.

2. Conclusion of the Panel Regarding Systemic Toxicity

The panel concluded that BZC produced little or no target organ toxicity and that the most appropriate dose level to use to define the highest no observed effect level (NOEL) for systemic toxicity was 12.5 mg/kg/day. This was the next to the highest dose level evaluated in both the rat and mouse thirteen-week studies. No systemic effects were observed in animals at this dose level, and even in animals at the highest dose level evaluated in these studies, i.e. 25 mg/kg/day, the slight systemic effects that were observed probably were secondary to severe skin irritation. This conclusion also is supported by the findings in the one-year dog and two-year rat studies in which BZC was administered by the dietary route of administration. In these studies, no systemic effects were observed in dogs at dose levels up to 12.5 mg/kg/day or in rats at dose levels up to 10 mg/kg/day. In the latter study the only treatment related effects observed at the two higher dose levels of 50 and 125 mg/kg/day were distended ceca (observed grossly) and thinning of the cecal wall (observed microscopically). Since these findings have been observed in germ free rats, the committee felt that, in the present instance, the antimicrobial properties of BZC may have acted to produce a similar condition. If this is the case, the committee concluded that the condition could be characterized as a physiological rather than toxicological response. The dermal carcinogenicity studies were not considered to be appropriate for defining the NOEL for systemic toxicity because skin irritation effects and dose volume limited the actual systemic doses to 1.5 mg/kg/day.

E. Skin Irritation

1. Data Reviewed and General Commentary

a. Laboratory animal studies

The data describing the effects of various concentrations of BZC evaluated in the three rat and three mouse dermal toxicity studies conducted as part of the NTP were reviewed from the draft reports which were provided to the panel. Summaries of these rat and mouse studies are provided in the Reference Volume behind Tabs 10 and 11, respectively.

In rats, dose solution concentrations ranging from 0.6 to 9.6% in ethanol were evaluated in a 16-day study; concentrations ranging from 0.156 to 2.5% in ethanol were evaluated in the 13-week

study; and concentrations ranging from 0.025 to 0.25% in ethanol were evaluated in the two-year study. In all cases, the dose solutions were prepared using 95% ethanol (USP Grade) and were applied five days per week. With the exception of the lowest concentration evaluated in the two-year study (0.025%) in which no irritation was observed, there was a concentration dependent increase in epithelial hyperplasia and inflammation in all studies. Also in all studies, more severe skin changes including necrosis and/or ulceration were observed at the highest concentrations.

In mice, dose solution concentrations ranging from 0.15 to 2.41% in ethanol were evaluated in the 16-day study; concentrations ranging from 0.05 to 0.8% in ethanol were evaluated in the 13-week study; and concentrations ranging from 0.006 to 0.06% in ethanol were evaluated in the two-year study. In all cases, the dose solutions were prepared using 95% ethanol (USP Grade) and were applied five days per week. With the exception of the lowest concentration evaluated in the two-year study (0.006%) at which no irritation was observed, there was a concentration dependent increase in epithelial hyperplasia with or without inflammation in all studies. In the 16-day and 13-week studies, more severe skin changes in the form of necrosis, ulceration or necrotizing inflammation were observed in animals at the higher concentrations.

The data from these rat and mouse studies demonstrate that BZC has the potential to produce skin irritation which is both concentration and time dependent. However, the fact that all applications were in the form of ethanol solutions limits the usefulness of the data for evaluating the potential skin irritation effects of BZC when used as an antibacterial agent in consumer hand soaps.

The panel also reviewed the following laboratory animal studies in which skin irritation was evaluated:

- A four-week study in which rabbits were administered dermally 2 ml of a 0.1% solution of BZC five days per week; The solvent used in this study was not specifically mentioned, but was assumed to be water. No signs of skin irritation were noted (Reference Volume, Tab 12).

- A study in which black mice received a single application of BZC in tricapylin at six dose levels ranging from 8.75 to 280 mg/kg; In animals at the two highest dose levels (140 and 280 mg/kg) severe blistering was observed. In animals at the two intermediate doses (35 and 70 mg/kg) more moderate local reactions occurred, and in animals in the two lowest doses no visible reactions occurred (Reference Volume, Tab 2).

Because of the large differences between the way BZC was administered in these studies and the use patterns being considered in this review, these studies also were of little value in evaluating the potential skin irritation effects of BZC when used as an antimicrobial agent in consumer hand soaps.

b. Human clinical studies

The panel reviewed the following human clinical studies in which skin irritation was evaluated:

- A study in which 0.1 ml of a five percent solution of BZC in water was applied under an occlusive patch for a period of 48-hours to the upper backs of 100 male volunteers; Approximately one-half of the volunteers showed irritant reactions defined as redness without vesiculation or infiltration. The irritation did not spread beyond the patch and decreased in intensity after 24 hours (Reference Volume, Tab 13).
- A study in which BZC (phemerol), either as a 0.2% aqueous solution or as a tincture, was evaluated as a preoperative skin antiseptic; Three hundred obstetric deliveries and 100 surgical cases were evaluated. No evidence of irritation was observed (Reference Volume, Tab 14).
- A human repeated insult patch test in which BZC was added to an aerosol antiperspirant or a deodorant formulation at a concentration of 0.12%; An occlusive patch technique was used in 50 volunteers. No evidence of

irritation was observed after either the eight induction or single challenge applications (Reference Volume, Tab 15, pages 91 through 93).

2. Conclusion of the Panel Regarding Skin Irritation

The results of these studies indicate that BZC has the potential to cause skin irritation, particularly at a high aqueous concentration and under exaggerated use conditions, i.e. 5%, under an occluded patch for 48 hours. At lower BZC concentrations (0.1 to 0.2%) in formulated skin products BZC appears to have little or no irritant effects.

All antimicrobial skin soap formulations containing BZC will have to be evaluated carefully for skin irritation in order to label the products properly and to avoid potential skin irritation effects.

F. Ocular Irritation

1. Data Reviewed and General Commentary

The panel reviewed the following studies which were conducted to evaluate ocular irritation:

- An ocular study in which different concentrations of BZC were instilled in the eyes of albino rabbits for the purpose of determining the highest concentration which did not produce irritation in three or more of the five test rabbits during the first hour after ocular instillation; This concentration was determined to be 0.03%. The vehicle used in this study was not specifically mentioned, but was assumed to be water (Reference Volume, Tab 12).
- An ocular study in which different aqueous concentrations of BZC were instilled into the eyes of albino rabbits for the purpose of determining the highest concentration for which corneal or iridic irritation was not present seven days after the initial ocular instillation; This concentration was determined to be 0.5% (Reference Volume, Tab 16).
- An ocular study in which a 0.1% solution of BZC (phemerol) was instilled into the eyes of albino rabbits two to three times per day for one to three months; The superficial layers of the cornea became thick and rough and slight vascularization of the corneal

stroma was noted. However, the deeper layers of the cornea and other intraocular tissues were not affected. The vehicle used in this study was not specifically mentioned, but was assumed to be water or saline (Reference Volume, Tab 17, page 1120).

- A study conducted by Lonza Inc. in which aqueous concentrations of BZC corresponding 1.0, 1.6 and 3.2% were evaluated in a standard Draize eye irritation test; At the 1.0% concentration no corneal opacity was observed and iridial and/or conjunctival irritation cleared within 14 days. At the 1.6 and 3.2% concentrations corneal opacity, iridial and conjunctival irritation were observed. With the exception of mild conjunctival irritation in one of the six rabbits, all irritation at the 1.6% concentration cleared within 14 days. At the 3.2% concentration corneal opacity and conjunctival irritation persisted through the end of the study, i.e. 21 days. A summary of these data are provided in the Reference Volume behind Tab 18.

2. Conclusion of the Panel Regarding Ocular Irritation

The findings in these studies indicate that BZC has the potential to produce eye irritation even at low concentrations, i.e. $< 0.1\%$. At higher concentrations ($\geq 1.0\%$) it has the potential to produce long lasting or possibly irreversible eye damage. Therefore, all antimicrobial skin soap formulations containing BZC will have to be evaluated carefully for eye irritation in order to label the products properly and to avoid potentially severe eye effects due to accidental ocular exposure.

G. Skin Sensitization

1. Data Reviewed and General Commentary

A draft report for a Magnusson-Kligman Maximization Test conducted with BZC by Lonza Inc. and a full report from a contact hypersensitivity study conducted with BZC by the NTP were available for review. Data from photoallergy studies conducted with ADBAC and DDAC also were considered. A detailed summary of the Lonza Inc. study is provided in the Reference Volume behind Tab 19. The full NTP report is provided in the Reference Volume behind Tab 20.

Aqueous concentrations of 1.0 and 2.0% were used for induction and challenge, respectively in the maximization test. In the NTP contact

hypersensitivity study, BZC concentrations of 1.0, 3.0 and 10.0% in 95% ethanol were used for induction and a 20% BZC concentration in 95% ethanol was used for challenge. No evidence of sensitization was observed in either study. In addition, ADBAC and DDAC were shown not to be photoallergens. Therefore, it is unlikely that BZC is a sensitizer.

Two other studies reviewed by the panel were human repeated insult patch test in which BZC was added to either an aerosol antiperspirant or a deodorant formulation at a concentration of 0.12%. In these studies, the occlusive patch technique was used and 50 volunteers received eight induction applications (four per week for two weeks) followed by a single challenge application two weeks later. No evidence of sensitization was observed with either formulation (Reference Volume, Tab 15, pages 91 through 93).

The latter studies provide some confirmatory evidence that BZC is not a sensitizer in humans at low use concentrations.

2. Conclusion of the Panel Regarding Skin Sensitization

While none of the studies that were reviewed indicate that BZC is an animal or human sensitizer, a large panel HRIPT should be conducted to demonstrate that BZC is not a sensitizer in humans at the higher concentrations scheduled to be used in the skin soap formulations.

H. Absorption (A), Distribution (D) and Excretion (E)

1. Data Reviewed and General Commentary

The abstract and discussion sections of a report prepared by the NTP for a "standard" ADE study conducted in rats and a report for a study in which the maternal and fetal absorption of BZC was evaluated following oral administration of ^{14}C -BZC were available for review. The pertinent sections of the NTP report are provided in the Reference Volume behind Tab 21. The report for the maternal/fetal absorption study is provided in the Reference Volume behind Tab 22.

In the NTP ADE study, it was shown that dermally administered BZC is absorbed readily through the skin of rats, distributed into tissues and excreted rapidly, primarily in the feces. In the maternal/fetal absorption study, low levels of radioactivity were found in the blood stream of the dams following oral administration of ^{14}C -BZC to pregnant rats on

gestation days 6 to 15. Variable and/or questionable levels of radioactivity were found in the fetuses.

2. Conclusion of the Panel Regarding Absorption, Distribution and Excretion

While it is well established that the skin of rats is much more permeable to most chemicals than is the skin of humans, given the high degree of permeability observed in the rat ADE study, BZC also may be able to penetrate the skin of humans to a significant degree. While the findings from the rat ADE studies do not raise any toxicological concerns on the part of the panel, this panel recommends using a dermal absorption factor of 50% for human risk assessment purposes.

III. Quantitative Risk Assessment

The panel performed a quantitative risk assessment in which the no observed effect level (NOEL) for the systemic toxicity derived from animal toxicology studies was compared to potential human exposure to BZC when used as an antimicrobial agent in consumer hand soaps. The following assumptions and/or values were used in this assessment:

Soap usage per day (90th percentile of human use = 10 times x 1.5 grams)	15 grams ¹
Maximum percentage of BZC in consumer hand soap formulations	5.0% ²
Percent of soap remaining on human skin after washing	1% ³
Human dermal absorption of BZC from hand soap formulations	50%
Average body weight of consumer	40 kg
NOEL from animal toxicology studies	12.5 mg/kg/day

¹ Personal communication with the Dial Corporation

² Personal communication with Lonza Inc.

³ Standard value used by FDA

Exposure Calculation

$$15g \times 0.05 \times 0.01 \times 0.50 \times \frac{1000mg}{g} = 3.75mg/person/day$$

$$\frac{3.75mg/person/day}{40kg} \times \frac{1}{person} = 0.09375mg/kg/day$$

Safety Factor Calculation

$$\frac{\text{NOEL from animal toxicology studies (mg/kg/day)}}{\text{Human exposure (mg/kg/day)}} = \frac{12.5mg/kg/day}{0.09375mg/kg/day} = 133$$

The above calculation provides a safety factor of 133. Since the assumptions which went into this calculation are considered to be conservative and protect the 90th percentile of human consumers, it is the opinion of the panel that an adequate margin of safety exists for the use of BZC as an antimicrobial agent in consumer hand soap.

Another potentially important factor which the panel discussed in evaluating the potential risk associated with the use of BZC as an antimicrobial agent in consumer hand soaps is the potential bioavailability of BZC when it is incorporated into a hand soap formulation. The reason that this was felt to be a potentially important factor is that in soap-type formulations BZC forms micelles with the other formulation components. While no information is available for the type of hand soap formulation in which BZC will be added for this particular application, it is well established that micelle formation significantly reduces the bioavailability of BZC as measured in terms of efficacy. For example, while BZC is an effective bactericidal agent as in a pure water or alcohol solution at a concentration of 500 ppm (0.05%), BZC concentrations as high as 50,000 ppm (5.0%) may be needed to obtain the desired efficacy in a soap-type formulation.

In addition to reducing efficacy, the micelle formation also may reduce the amount of BZC that would be absorbed through the human skin, thereby reducing the systemic exposure that would occur with the type of application being considered.

Further, since the mechanisms by which BZC produces its antimicrobial effects (disruption of the permeability of cellular membranes; protein denaturation; inhibition of enzymes and oxidative processes; effects on activating ions; and interference with growth and reproduction) also are likely to be the mechanisms by which BZC exerts its irritant

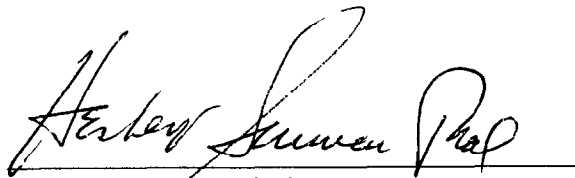
effects, the proposed hand soap formulations are not expected to be as irritating to the human skin as pure aqueous or alcohol solutions have been shown to be in animal tests.

IV. Panel Conclusions and Recommendations

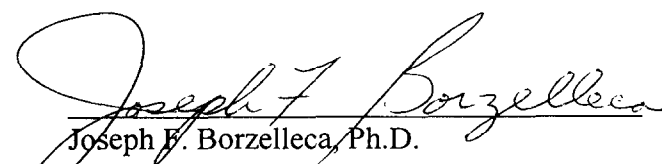
The panel concluded that the database was adequate to evaluate the safety of using BZC as an antimicrobial agent in consumer hand soap products and that this database supports the opinion that this use pattern will not be associated with any unacceptable risks. The additional studies the panel recommends are a human repeated insult patch test (HRIPT) and a rabbit eye irritation test with a representative finished formulation. The HRIPT should be conducted at the maximum nonirritating concentration of BZC as defined in a skin irritation screen. For the definitive HRIPT, a panel of 150 volunteers is recommended. The rabbit eye irritation test should be conducted according to the standard Draize procedure at the maximum BZC concentration that is expected to be present in consumer hand soap formulations.

Other areas of investigation in which the panel feels useful information would be obtained are studies designed to define the percent of BZC absorption through human skin and to explore the relationship between micelle formation and bioavailability, particularly as it relates to absorption. The panel feels that this information would be useful because it has the potential to demonstrate that there is a much higher level of safety associated with the use of BZC in soap formulations than is indicated by the quantitative risk assessment that was performed using the assumption that 50% of the residual BZC would be absorbed into the systemic circulation.

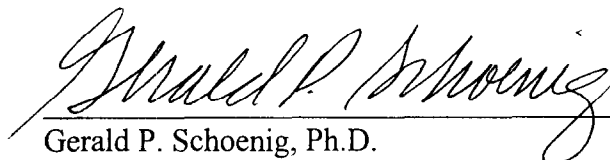
V. Signatures of the Panel Members


Herbert Blumenthal Ph.D.

December 13, 1995
Date


Joseph F. Borzelleca, Ph.D.

12 December 1995
Date


Gerald P. Schoenig, Ph.D.

December 11, 1995
Date

Expert Panel Review of Benzethonium Chloride

Reference Materials

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**EXPERT PANEL REVIEW OF
BENZETHONIUM CHLORIDE**

Volume II: Reference Documents

Panel Reviewers:

Herbert Blumenthal, Ph.D.
Joseph F. Borzelleca, Ph.D.
Gerald P. Schoenig, Ph.D.

Panel Coordinator

Mr. Eliot Harrison

December 13, 1995

Expert Panel Review of Benzethonium Chloride
Reference Materials
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Summaries of Completed Studies on DDAC	Tab 25

Skin Changes: In-life: There was reddening of the skin for the males in all dose groups and for the females in the 0.15 mg/kg/day dose group. Crusting was observed for females at 0.5 mg/kg/day. No information is provided in the report regarding the females in the high-dose group.

Necropsy: Information on skin changes noted at necropsy were not provided in the laboratory report.

Necropsy: Necropsy findings (or lack thereof) for systemic organs and tissues were not discussed in the laboratory report.

Organ Weight: There were no toxicologically significant changes in liver or kidney weights.

**Histopathology-
Neoplastic lesions:** There were no neoplastic lesions observed for animals in this study.

Nonneoplastic lesions: There were no nonneoplastic lesions in this study that indicated an effect of the test substance on systemic organs or tissues.

Dose-related increases in the incidence of animals with epithelial hyperplasia were observed at the site of application for males at the 0.5 mg/kg/day and 1.5 mg/kg/day dose levels and for females at the 1.5 mg/kg/day dose level. Other lesions at the site of application occurred sporadically, or were observed also in the control group, and were not attributed to treatment with the test substance.

There were no clear differences between the lesions noted at the 15- and 24-month sacrifices.

Conclusions

Repeated dermal application of benzethonium chloride for two years resulted in skin irritation that ranged in severity from minimal at the low dose (0.15 mg/kg/day) to mild at the high dose (1.5 mg/kg/day). Benzethonium chloride did not result in systemic toxicity or oncogenicity at any dose level. The NOEL in this study for systemic toxicity and oncogenicity was at least 1.5 mg/kg/day.

Two-Year Dermal Toxicity Study With Benzethonium Chloride in Mice

General Information

Reference: NTP Draft Technical Report
Report Date: June 22, 1994
Testing Laboratory: Battelle Columbus Laboratories

Study Design

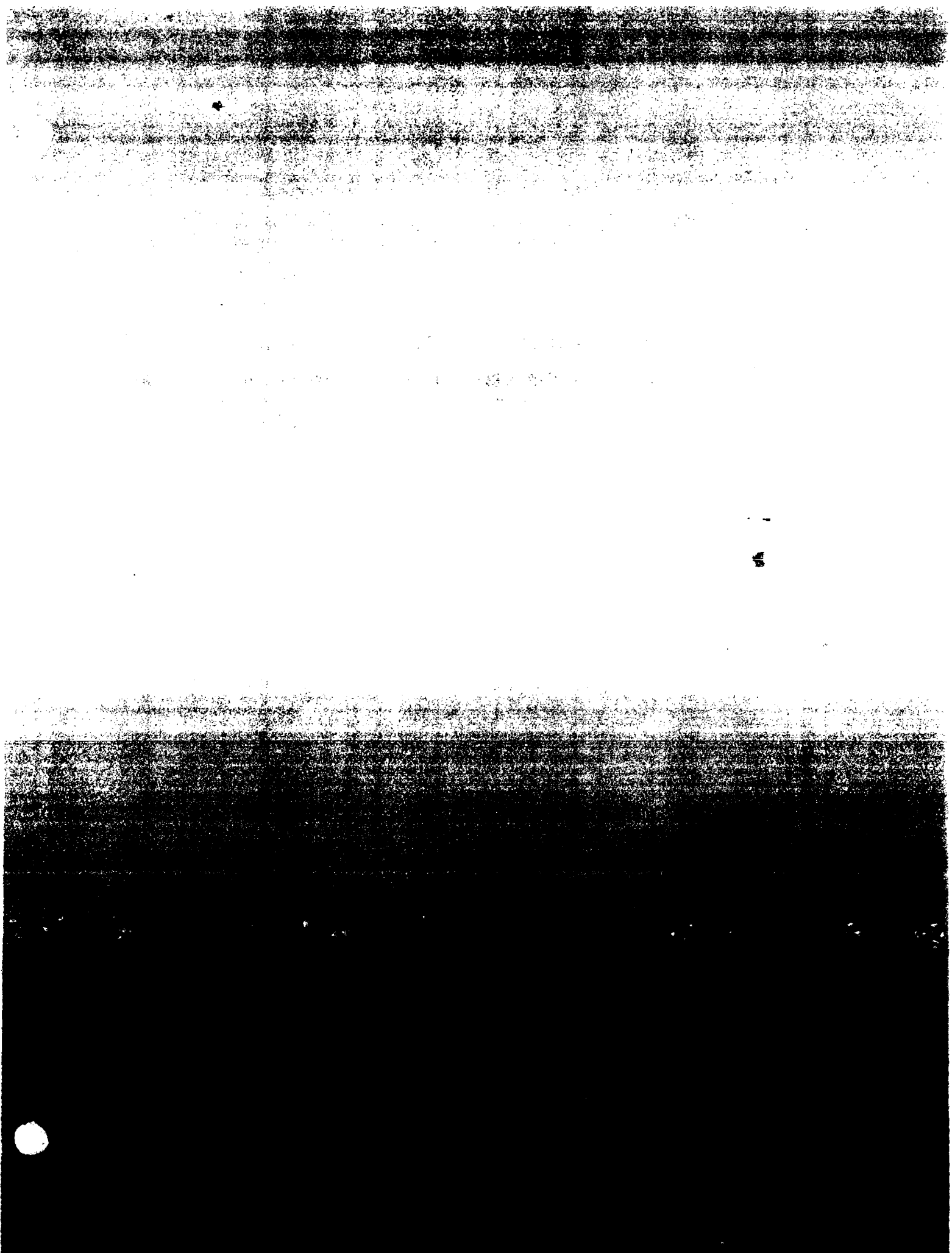
Test System: Male and female B6C3F₁ mice
Age at Start of Test: 40 days
Dose Levels: 0, 0.15, 0.5, 1.5 mg/kg/day.
Dose Solution Concentrations: 0, 0.006, 0.02, 0.06 % in 95% ethanol, USP
Dose Volume: \approx 131 μ l
Dose Route and Regimen: Topical, to dorsal interscapular area, unoccluded;
five applications per week for 65 or 104 weeks
Number of Animals per Group: 60 males and 60 females
Experimental Evaluations: i. Twice daily observations for mortality and
clinical signs of toxicity; body weight recorded
weekly through week 10, once during week 12
and monthly thereafter; clinical observations
recorded monthly;
ii. Necropsy;
iii. Organ weight: Kidney and liver weight for
6 to 10 mice/sex/group at 15-month sacrifice;
iv. Complete histopathology on all control and
1.5 mg/kg/day mice; examination of skin from
application sites and undosed sites from all dose
groups at 15- and 24-month sacrifices.

Results

Mortality and Clinical

Observations: There were no treatment-related changes in survival or clinical signs of systemic toxicity.

Body Weight: There were no treatment-related changes in body weight.



Two-Year Dermal Toxicity Study With BZC in Rats

Skin Changes:	In-life: Reddening of the skin was observed for all dose groups. Crusting was observed for males at doses ≥ 0.5 mg/kg/day and for females at a dose level of 1.5 mg/kg/day. Necropsy: Information on skin changes noted at necropsy were not provided in the laboratory report.
Necropsy:	Necropsy findings (or lack thereof) for systemic organs and tissues were not discussed in the laboratory report.
Organ Weights:	No treatment-related changes were observed in liver or kidney weights.
Histopathology- Neoplastic lesions:	There were no neoplastic lesions observed for animals in this study.
Nonneoplastic lesions:	There were no nonneoplastic lesions in this study to indicate an effect of the test substance on systemic organs or tissues. Minimal to moderate dose-related increases in epithelial hyperplasia were observed at the site of application for males and females at dose levels ≥ 0.5 mg/kg/day. Sebaceous gland hyperplasia was sometimes observed in association with more severe cases of epithelial hyperplasia. Epidermal ulceration was observed frequently for females at the 1.5 mg/kg/day dose level and for one male at the 1.5 mg/kg/day dose level. There were no clear differences between the lesions observed at the 15- and 24-month evaluations.

Conclusions

Repeated dermal application of benzethonium chloride for two years resulted in skin irritation in animals at all dose levels that ranged in severity from minimal in the low-dose (0.15 mg/kg/day) group animals to moderate in the high-dose (1.5 mg/kg/day) group animals. Benzethonium chloride did not result in systemic toxicity or oncogenicity in animals at any dose level. The NOEL in this study for systemic toxicity and oncogenicity was at least 1.5 mg/kg/day.

Two-Year Dermal Toxicity Study With Benzethonium Chloride in Rats

General Information

Reference: NTP Draft Technical Report
Report Date: June 22, 1994
Testing Laboratory: Battelle Columbus Laboratories

Study Design

Test System: Male and female F344/N rats
Age at Start of Test: 45 days
Dose Levels: 0, 0.15, 0.5, 1.5 mg/kg/day
Dose Solution Concentrations: Males: 0, 0.025, 0.083, 0.25 %
in 95% ethanol, USP
Females: 0, 0.015, 0.05, 0.15 %
in 95% ethanol, USP
Dose Volume: $\cong 317\mu\text{l}$
Dose Route and Regimen: Topical, to dorsal interscapular area, unoccluded;
five applications per week for 104 weeks
Number of Animals per Group: 60 males and 60 females
Experimental Evaluations: i. Twice daily observations for mortality and
clinical signs of toxicity; body weight recorded
weekly through week 10, once during week 12
and monthly thereafter; clinical observations
recorded monthly;
ii. Necropsy;
iii. Organ weight: Kidney and liver weights for
4 to 9 rats/sex/group at 15-month interim
sacrifice;
iv. Complete histopathology on all control and
1.5 mg/kg/day rats; examination of skin from
application sites and undosed sites from all dose
groups at 15- and 24-month sacrifices.

Results

Mortality and Clinical

Observations: There were no treatment-related changes in survival or clinical signs of systemic toxicity.

Body weight: There were no treatment-related changes in body weight.

FINAL REPORT

Contract PH-43-67-677

Project C-173

Period Covered by this Report:

May 23rd through August 22nd, 1968

Distribution:

45 copies of Progress Report and
20 copies of Abstract to Mr. Damian Crane

4 copies - For Internal Use Only

Report Compiled by:

Freddy Homburger, M.D.
President and Director
Bio-Research Consultants, Inc.
9 Commercial Avenue
Cambridge, Massachusetts 02141

Date of this Report:

September 15th, 1968

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ABSTRACT

HOMBURGER, FREDDY (Bio-Research Consultants, Inc., Cambridge, Mass.) The purpose of this study was to determine: A. Whether seven test compounds (Thimerosal, ethylene chlorohydrin, Methyl Paraben, Phenol Red, ethylene glycol, Benzethonium chloride and pyridine) cause malignant tumors in mice when given subcutaneously and when four pooled injection sites are transferred into one secondary host, a technique used to accelerate the biological effects. --All seven compounds were devoid of demonstrable carcinogenic effects, in contrast to 3,4,9,10-dibenzpyrene which caused fibrosarcomas under the same conditions in from 8 to 17 weeks after site transfer. B. Whether the test compounds when given intravenously to mice in single or repeated injections accelerate the development of spontaneous lung adenomas in CF 1 and in A mice. --No acceleration of lung adenoma formation was observed with any of the test compounds in single or multiple injections in either mouse strain. This is in marked contrast to the acceleration of tumor formation by 3,4,9,10-dibenzpyrene. C. Whether the test compounds are co-carcinogenic in mice when given subcutaneously following a standard carcinogen. --In spite of published evidence that the two-stage concept of carcinogenesis prevails in subcutaneous tissue, these studies were inconclusive, since the positive control (croton oil) failed to show co-carcinogenic activity under the same experimental conditions. An incidental consistent observation was the decoloration of the fur of black mice caused at and near the site of injection by subcutaneous administration of Benzethonium chloride. This same substance also inhibited the formation of tumors (in the co-carcinogen studies) following subcutaneous injection of 3,4,9,10-dibenzpyrene.

--Author

RECAPITULATION OF MISSION
(Quoted from Contract Proposal)

"1. Attempt to Obtain Tumor Formation in Shorter Periods of Time: This protocol follows procedures outlined in the attached manuscript, which explains our new techniques to shorten the latent period between carcinogen injection and tumor formation. One hundred C57BL/6 mice are used per group and receive injections subcutaneously into the groin. At the end of 8 and 12 weeks, the injection sites of each group are excised, pooled and homogenized in Ringer's solution, and the pooled material is transferred into 25 mice (an increase in amount of transferred tissue by a factor of 4) of the same strain and age. The secondary hosts are palpated weekly and any tumors appearing are treated as in a), above.

2. Intravenous Injection into A/Heston Jax Mice of Test Substances and Observation of Lung Adenomas: According to Harold L. Stewart, the lung tissue of A/Heston mice is the most sensitive indicator for carcinogens available. When known carcinogens are injected subcutaneously into A/Heston mice, greatly increased numbers of adenomas can be counted on their lung surfaces before sarcomas form at the injection site. Upon intravenous injection of benzpyrene and other known carcinogens, the number of lung adenomas increases in proportion to the administered dose. We have confirmed in our own laboratory both of these observations and believe with Stewart that a substance that fails to cause an increase of adenomas of A/Heston mice upon intravenous injection is extremely unlikely to be a carcinogen under other conditions.

Fifty A/HeJ mice will be used per compound and for a vehicle control. At three months of age, they will receive a single intravenous injection of the test substances at the highest tolerated dose (dose-finding must first be done by the intravenous route for each compound). Three to six months later the mice will be killed, their lungs inflated with formaldehyde, and the adenomas on the lung surface will be counted under a x 15 dissecting microscope. Spot checks can be made in some animals to determine whether the experiment could be terminated earlier. Histological studies will be made in selected lung specimens.

3. Studies on Co-carcinogenesis (Promotion): Extensive experience has accumulated in our laboratory on the carcinogenic effect of benzo(rst) pentaphene (old nomenclature: 3,4,9,10-dibenzpyrene), a potent carcinogen that has the advantage of largely remaining at the original site of its injection. Fifty C57BL/6 male mice will be used per group for each test material. They will be randomized as in experiment 1a, above, and after

acclimatization, they will receive 12.5 mμ of benzo(rst)pentaphene in the region of the groin. Mice of the control group will receive the vehicle twenty-four hours later into the same site. This will be repeated at weekly intervals on three occasions. In the test groups, these "promoting" injections will be done in the same vehicle at similar intervals using the test compounds at the doses used in 1a, above. An additional control group will receive promoting injections of croton oil in the same vehicle in the maximum tolerated dose. Weekly palpations will be done in these mice and any palpable tumors will be sectioned for histological examination."

Modification of Experiments

We were authorized to add one group of mice in the subcutaneous co-carcinogen study at one-half of the initial dose of Benzethonium chloride (0.35 mg).

Furthermore, in Supplemental Agreement No. 1 of January 2nd, 1968, the following additional studies were authorized:

"a. Inject intravenously 8 groups of 20 CF 1 female mice on days 0, 30 and 60, with examination of the lungs three months after the last injection.

b. Inject intravenously (single dose) 8 groups of 50 A/Jax female mice."

Finally on May 23rd, the duration of this contract was extended to August 22nd, 1968.

SOURCES OF COMPOUNDS; DOSE-FINDING EXPERIMENTS

On June 6th, 1967, the following substances for testing were received from N.I.H.:

Table 1

List of Compounds and Their Sources

Compound	Bio Code	Wt. (gms)	Manufacturer
Thimerosal	1	3.0	Eli Lilly & Co.
Methyl Paraben	2	9.0	Tenneco Chem. Inc. (Heyden Div.)
Benzethonium chloride	3	10.0	Rohm & Haas
Pyridine*	4	30.0	Fisher Scientific Co.
Phenol Red	5	25.0	Allied Chem. (Nat. Aniline Div.)
Ethylene glycol	6	270.0	Fisher Scientific Co.
Ethylene chlorohydrin	7	6.5	Eastman Organic Chemicals

* The first shipment of pyridine was replaced because of corrosion of the stopper in the first bottle.

Determination of Highest Tolerated Subcutaneous Doses

The maximum single tolerated dose of these compounds was estimated by subcutaneous (s.c.) route in C57BL/6 male mice thirteen weeks of age. The mean body weight of 168 mice (8 mice/group) used in preliminary experiments was 20.0 (range: 17 gm to 25.4 gm).

The data given to us by Dr. Singer of N.I.H. for the LD/50's for these substances were used as a basis for dose-finding experiments. The maximum tolerated dose was assumed to be about 50% or less of these LD/50's. In twenty dose-finding experiments using eight mice for each, the following doses were established as the maximum tolerated amount for repeated injections.

Table 2

Maximum Tolerated Doses for Repeated Subcutaneous
Injections in C57BL/6 Male Mice

Thimerosal (suspension)	10 mg/kg (10 mg/kg- 80 mg/kg) *
Methyl Paraben (solution)	125 mg/kg
Benzethonium chloride (suspension)	35 mg/kg (17.5 mg/kg- 180 mg/kg)
Phenol Red (saturated suspension at about 600 mg/kg)	
Ethylene glycol (emulsion)	1300 mg/kg (1,300 mg/kg-7,800 mg/kg)
Ethylene chlorohydrin (solution)	60 mg/kg (60 mg/kg- 180 mg/kg)
Pyridine (solution)	300 mg/kg (300 mg/kg- 865 mg/kg)
Croton oil (solution)	0.1 % (0.1 % - 25 %)

* The figures in parentheses indicate the dosage range tested.

The vehicle for all substances was tricaprylin (Eastman Kodak Co., Rochester, New York).

Observations on Toxicity and Side Effects by Subcutaneous Route
Using Maximum Tolerated Doses

Four days after injection of ethylene glycol, seven animals were lost during the weekend. This may have been due to factors other than the test compound. All animals in the other groups survived and appeared in good condition.

The following gross observations were made at the injection sites:

Thimerosal - extensive inflammatory reaction at the injection site with large, semi-solid, palpable cystic masses. Three animals showed slight ulcera-

tions at the injection site.

Methyl Paraben - Small, ill-defined, soft cysts in majority of animals; later on, small ulcerations which healed.

Phenol Red - Cysts similar to those in the Methyl Paraben group; Phenol Red observed to be excreted in the urine.

Croton oil - A few small ulcerations were observed.

Pyridine - Minor ulcerations which healed.

Benzethonium chloride - Some large ulcers which healed within approximately four weeks and the delayed changes described below.

Ethylene glycol, ethylene chlorohydrin and tricaprylin showed no effect.

Observations on Depigmentation of Fur by Subcutaneously-
Injected Benzethonium Chloride

In all of eight animals (C57BL/6 mice) that were given 70 mg/kg of Benzethonium chloride subcutaneously in dose-finding experiments, white spotting of the fur was noted thirty-four days later near the site of injection. Five out of eight animals receiving 35 mg/kg at the same time showed a similar spotty depigmentation of their fur. When the bleached fur was plucked and allowed to regrow, the new hair in all plucked animals grew again as white fur.

Groups of three male C57BL/6 mice were painted by means of a Camel's hair brush with the following doses of Benzethonium chloride: 280 mg/kg, 140 mg/kg, 70 mg/kg, 35 mg/kg, 17.5 mg/kg, 8.75 mg/kg and, as a control, tricaprylin alone. There were severe local blistering reactions at the two highest dose levels, less marked local reactions at the 70 and 35 mg/kg levels, and no visible reaction with 17.5 and 0.75 mg/kg. No immediate bleaching occurred. Ten days later, there was spotty depigmentation of fur at the site of painting in two of the mice that had received 140 mg/kg. Hair exposed in vitro for forty-eight hours was not bleached by the concentrations of Benzethonium chloride used in the painting experiment.

Doses for Intravenous Injections; Observations on Acute Toxicity

It was found that the maximum tolerated subcutaneous dose of all compounds was adequately tolerated via the intravenous route and, therefore, the doses used in subcutaneous injections were also used for single and multiple intravenous injections. The gross observations made on animals having received

single intravenous injections of the various compounds are shown below.

Table 3

Acute Toxicity of Seven Compounds when Given Intravenously to A/Jax Mice
in Groups of Six Animals

The intravenous injection in Ringer's solution of that dose which, when given subcutaneously, was the maximum tolerated dose, caused the following observations:

Ethylene glycol	26 mg/mouse	no effect
Thimerosal	0.2 mg/mouse	no effect
Ethylene chlorohydrin	1.2 mg/mouse	no effect
Benzethonium chloride	0.7 mg/mouse	half of mice died
Benzethonium chloride	0.35 mg/mouse	no effect
Pyridine	6 mg/mouse	palpitations for one minute; shock; recovery after forty-five minutes
Methyl Paraben	2.5 mg/mouse	gasping respiration; shock; recovery after ninety minutes
Phenol Red	12 mg/mouse	two mice died in first group; four recovered in a second group; four out of five died
Phenol Red	6 mg/mouse	two out of nine mice died
Phenol Red	3 mg/mouse	six mice survived after episode of tachypnea; red color of skin disappeared after twenty-four hours

REPORT ON ATTEMPT TO OBTAIN TUMOR FORMATION
IN SHORTER PERIODS OF TIME

Method

As outlined in the original proposal, the method used was that described by Homburger and Treger in Cancer Research (27:1205-1213, 1967). One hundred C57BL/6 males, seven weeks old, received injections of the test compounds into the groin in the doses shown in Table 1. The medium used to dissolve or suspend the test compounds was 0.1 ml of tricapylin (Eastman Kodak Co., Rochester, New York).

Table 4

Doses of Compounds Used in Injection Site Transfer Studies;
Numbers of Survivors

Compound	Dose / Mouse	Survivors
Thimerosal	0.2 mg	21/25
Ethylene chlorohydrin	1.2 mg	24/25
Methyl Paraben	2.5 mg	12/25
Phenol Red	12 mg	25/25
Ethylene glycol	26 mg	15/25
Benzethonium chloride	0.7 mg	25/25
Pyridine	6 mg	25/25
<u>Positive Control</u>		
Dibenzpyrene	25 mg	24/25
<u>Negative Control</u>		
Tricaprylin	0.1 mg	23/25

Five weeks after these injections, the injection sites were excised, minced in 6 ml of Ringer's solution and pooled. The resulting tissue brei was divided into twenty-five equal portions, each of which was injected subcutaneously into one secondary host (C57BL/6 male of same age as primary host [twelve weeks]). The injection sites in the secondary hosts were palpated weekly beginning one week after transfer of the pooled injection sites.

In the positive controls injected with dibenzpyrene, the first palpable tumors appeared eight weeks after transfer of the sites (four tumors or 16%). Ninety-six percent of the animals had palpable tumors seventeen weeks after transfer of the sites. This was exactly as in earlier experiments on injection site transfers with dibenzpyrene (Figure 1). Whenever a palpable tumor was found, the animals were killed and the tumor was preserved for histological studies. No palpable tumors were observed in any of the other groups in this experiment.

Twenty-three weeks after the injection of the compounds into the primary hosts and eighteen weeks after transfer of the injection sites into secondary hosts, all animals were sacrificed. All injection sites were excised and preserved for histological studies.

Gross autopsies were made of all animals, and abnormal organs were prepared for histological study. All carcasses were preserved. Table 4 lists the numbers of survivors after site transfer in all experiments.

Comments on Mortality

The only significant mortality observed was in Methyl Paraben and in the ethylene glycol groups. In the Methyl Paraben group eight weeks after the site transfer, six animals in different cages were found dead without any apparent reason. The carcasses were too autolyzed for study. Four weeks later, another six animals were dead under similar circumstances.

In the ethylene glycol group, ten animals in one cage were found dead eight weeks after the transfer of injection sites. These animals had been reported "in poor shape" one week earlier. Again, the carcasses were too far autolyzed for study. There appears to be a cage factor in this particular instance. However, a similar loss of animals had previously occurred in dose-finding experiments on ethylene glycol.

Five injection sites of each of the groups were taken at random and processed for histological examination on hematoxylin-eosin-stained sections. The histological findings were remarkably similar in all of these groups. They showed granulation tissue forming multiple granulomas with numerous giant cells scattered throughout the subcutaneous tissue. There were strands of scar tissue and numerous cysts, either lined by single layers of endothelium-like cells or by granulation tissue with numerous giant cells, often containing cholesterol clefts. Some of the cysts were filled with amorphous, granular or reticular, pink staining material. There were no instances where fibroblasts in the granulation tissue and scar tissue even remotely suggested malignant transformation. In the tricapyrylin controls, similar foreign body reactions were seen. All of the palpable tumors of the dibenzpyrene series proved to be fibrosarcomas. (Figures 2 and 3 show photomicrographs of some transferred injection sites. Figure 4 is an example of a pathology report.)

The only groups wherein any pathology was noted outside of the injection sites were:

- a) the positive control (dibenzpyrene) - one enlarged liver showing congestion and distention of bile ducts and sinusoids;
- b) In the Benzethonium chloride group, there were four instances of enlarged spleens and livers. These were found on histological examination to be aging changes with probable amyloidosis. There was also one congested liver.
- c) In one animal of the Methyl Paraben group, there was one enlarged spleen similar to those described above.

These changes do not appear to be significant.

In the animals receiving Benzethonium chloride, all of the animals receiving the original injection showed decoloration of the fur, as described above. In the secondary hosts, no change of color was noted in the fur.

Conclusions

These studies suggest that none of the tested compounds can be demonstrated to be carcinogenic under the conditions of these experiments.

REPORT ON USE OF "THE MOST SENSITIVE SYSTEM" FOR CARCINOGEN DETECTION: INTRAVENOUS INJECTION INTO A/Heston AND CF 1 MICE AND OBSERVATION OF LUNG ADENOMAS

The methods used in this study were those described by Heston and Schneiderman in Science (117:109-111, 1953), and slightly modified by us (Lung Tumours in Animals, Proceedings of the Third Quadrennial International Conference on Cancer held at the University of Perugia, June 24-29, 1965, pp. 527-536).

Three groups of experiments were done. In the first two sets of experiments, single intravenous (tail vein) injections were made into groups of fifty CF 1 female and fifty A/Jax female mice, and in the third group, intravenous injections were made repeatedly (seven injections at monthly intervals) into groups of twenty CF 1 female mice. At the end of twenty-eight weeks, the mice were sacrificed, the lungs inflated with formaldehyde and inspected under a dissecting microscope for lung tumors visible on the lung surfaces. Histological sections were taken of some of the tumors seen (Figures 5-10).

The results are shown in Tables 5, 6 and 7.

The analysis of these data, based on Table II, Publication No. 749, National Academy of Sciences, National Research Council, shows that in this "most sensitive" system, none of the treated substances is carcinogenic for A/Jax or CF 1 mouse lungs. On the other hand, the low dose of 0.05 mg of dibenzpyrene was significantly carcinogenic at the 5% probability level when tested in CF 1 mice, as were the higher doses in the A/Jax mice.

CO-CARCINOGEN STUDY

Groups of fifty C57BL/6 male mice, randomized by weight so that the average body weight of the groups ranged from 22.1 to 22.4 gm, received 12.5γ of benzo(rst)pentaphene (3,4,9,10-dibenzpyrene [DBP]) in tricaprylin subcu-

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Table 5

Intravenous Injections and Lung Tumors in Groups of 50 CF 1 Female Mice;
Results after 28 Weeks

Date started	Compound	Dose/mouse (mg)	No. survivors	Animals with tumors (%)	No. tumors/mouse (average among those with tumors)
9/25/67	Thimerosal	0.2	49	10.2	1.0
10/2/67	Ethylene glycol	26.0	48	10.4	1.0
10/10/67	Pyridine	6.0	46	10.9	1.2
10/16/67	Ethylene chlorohydrin	1.2	46	10.9	1.0
10/23/67	Benzethonium chloride	0.35	46	19.5	1.1
10/31/67	Phenol Red	6.0	44	6.8	1.1
10/31/67	Methyl Paraben	2.5	44	21.7	1.4
<u>Negative Control</u>					
10/2/67	Ringer's (0.2 cc)	---	48	14.5	1.0
<u>Positive Controls</u>					
10/17/67	DBP	0.05	44	34.5	3.6
11/28/67	DBP	0.1	49	85.7	11.2
10/19/67	DBP	0.5	43	81.4	38

To be statistically significantly different from the tumor incidence in the negative controls (14.5%), the incidence in a treated group at the 1% probability level would have to be 40%; at the 5% probability level, the incidence would have to be 31%.

Table 6

Intravenous Injections and Lung Tumors in Groups of 50 A/Jax Female Mice;
Results after 28 Weeks

Date started	Compound	Dose/mouse (mg)	No. survivors	Animals with tumors (%)	No. tumors/mouse (average among those with tumors)
10/26/67	Phenol Red	6.0	44	23	1.1
10/26/67	Methyl Paraben	2.5	44	14	1.3
11/3/67	Ethylene glycol	26.0	41	19.5	1.1
11/3/67	Benzethonium chloride	0.35	39	25.6	1.1
11/8/67	Pyridine	6.0	42	19	1.0
11/8/67	Thimerosal	0.2	43	3.3	1.2
11/14/67	Ethylene chlorohydrin	1.2	45	22.2	1.0
<u>Negative Control</u>					
10/24/67	Ringer's	0.2 cc	48	14	1
<u>Positive Controls</u>					
11/17/67	DBP	0.05	47	68.1	8.7
11/16/67	DBP	0.1	48	83.3	19.8

Statistical significance considerations same as in Table 2.

Table 7

Repeated Intravenous Injections and Lung Tumors in Groups of 20 CF 1 Female Mice; Results after 28 Weeks

Date started	Compound	Dose/mouse (mg)	Total dose (mg)	No. survivors	Animals with tumors (%)	No. tumors/mouse (average among those with tumors)
11/21/67	Methyl Paraben	2.5	17.5	20	20	1.2
11/21/67	Phenol Red	6.0	42	18	11	1.0
11/21/67	Thimerosal	0.2	1.4	19	5.3	1.0
11/21/67	Benzethonium chloride	0.35	2.45	13	23	1.3
11/22/67	Ethylene chlorohydrin	1.2	8.4	18	28	1.2
11/22/67	Ethylene glycol	26.0	182	20	15	1
11/22/67	Pyridine	6.0	42	20	15	1
11/22/67	Ringer's	---	1.4 ml	18	11.1	1

To be statistically significantly different from the Ringer's controls (11%), the tumor incidence in any treated group would have to be 45% at the 5% probability level.

No positive controls were included, since extensive experience has taught us that with multiple injections of known carcinogens, the tumor incidence is 100% long before twenty-eight weeks elapse.

taneously and twenty-four hours later, the test compound in the same site. Additional injections of the test compound were made seven and fourteen days later, except in the case of Benzethonium chloride (Compound #3, Table 8) where only two injections were made. An additional series of Benzethonium chloride at 0.35 mg/mouse was started later after authorization had been obtained. Groups of mice received two injections of 70 mg each, the second group having received three injections of 35 mg each.

The injection sites were palpated weekly and tumors noted when 1 cm in diameter. Table 8 shows the results (also illustrated in Figure 11). The animals were killed at the end of the 29th to 31st weeks of the experiment. Histological examination showed that all tumors studied were the typical fibrosarcomas as usually produced by dibenzpyrene.

The outcome of this study is inconclusive as to co-carcinogenic effects. This is so because croton oil failed to have any co-carcinogenic action under the present experimental conditions. This renders less significant the observation that

Table 8

Co-carcinogen Study - Vehicle: Tricaprylin

Compound	Dose/mouse/ injection (mg)	Date started	Weeks after start	No. Animals		Cumulative no. tumors	Cumulative % tumors	Remarks
				Initial	Present survivors			
Control - DBP	0.025	6/22/67	31	50	48	25	52.0	No ulcerations
Control - (DBP) + Croton Oil	0.025 0.1	6/22/67	31	50	45	22	48.8	No ulcerations
Benzethonium chloride	0.7	7/5/67	29	50	41	14	34.1	All mice 'Pinto'; no ulcerations †
Benzethonium chloride §	0.35	8/17/67	23	50	38	0	0.0	97.4% 'Pinto'
Ethylene chloro- hydrin	1.2	7/5/67	29	50	47	20	42.5	No ulcerations
Pyridine	6	7/5/67	29	50	46	19	41.3	No ulcerations
Phenol Red	6	6/21/67	31	50	46	21	45.6	No ulcerations
Thimerosal	0.2	6/21/67	31	50	45	18	40.0	No ulcerations
Methyl Paraben	2.5	6/21/67	31	50	49	26	53.0	No ulcerations
Ethylene glycol	26.0	6/21/67; 6/26/67	31	70*	49	19**	38.9	No ulcerations

0.1 ml of 0.1% solution of croton oil in tricapylin.

† 'Pinto' refers to decoloration of fur previously reported.

§ Additional group of mice receiving one-half of the maximum tolerated dose of Benzethonium chloride.

* Because ten mice died shortly after the first injection, an additional twenty mice were injected, beginning six days after the first experiment.

** Average of two groups

none of the test compounds were co-carcinogenic.

There is a body of literature which suggests that the two-phase concept of carcinogenesis (initiation and promotion) is valid in the subcutaneous site. The pertinent publications are the following:

1. Sall, R.D. and Shear, M.J., Studies in Carcinogenesis. XII. Effect of the Basic Fraction of Creosote Oil on the Production of Tumors in Mice by Chemical Carcinogens. J. Nat. Cancer Inst. 1(1):45-55, 1940.
2. Cabot, S., Shear, N., Shear, M.J. and Perrault, A., Studies in Carcinogenesis. XI. Development of Skin Tumors in Mice Painted with 3:4-Benzpyrene and Creosote Oil Fractions. Am. J. Pathol. XVI(3):301-312, 1940.
3. Klein, M., The Action of Croton Oil in the Induction of Sarcomas in Mice. J. Nat. Cancer Inst. 11(4):843-848, 1951.

It is possible that the dose of croton oil used in the present experiment (0.1 mg) was too high, Klein observed positive co-carcinogenic effects with 0.004 mg of croton oil.

It is suggested that studies on subcutaneous co-carcinogenesis be repeated with the purified or synthetic phorbol esters now available from the work of Hecker (The Tumor-Promoting Principles from Croton Oil, In Progress in Experimental Tumor Research, F. Homburger, Ed., Vol. 12, S. Karger AG, Basel/New York, 1969) and Van Duuren (Tumor-Promoting Agents in Two-Stage Carcinogenesis, In Progress in Experimental Tumor Research, F. Homburger, Ed., S. Karger AG, Basel/New York, 1968) which, while more potent co-carcinogenic agents, are less irritating and less toxic.

COMMENTS ON BENZETHONIUM CHLORIDE

One interesting observation is the significant inhibiting effect of Benzethonium chloride upon tumor formation. This suggests that Benzethonium may be a cytotoxic compound as could also be deduced from its bleaching effect on hair.

Depigmentation of hair can occur in areas overlaying healing wounds or inflammation. In subsequent growth cycles, such hair will grow again with its natural color.

According to Dr. Herman B. Chase of Brown University, Providence, Rhode Island, who is an expert in this field of hair pigmentation and de-pigmentation

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(vide pp. 229-237, In The Biology of Hair Growth, Montagna and Ellis, Eds., Academic Press, 1958), the phenomenon which we have observed with Benzethonium chloride is rare, difficult to explain, and calls for additional research in depth. This could well be a radiomimetic effect such as has been described for nitrogen mustards.

We quote the following two paragraphs from A. Haddow's chapter In The Pathology of Cancer, F. Homburger, Ed., Hoeber-Harper, New York, 1959 (Sec. Ed.), p. 604:

"From all the evidence available it appears that the main primary action of the nitrogen mustards is exerted during the "resting stage", that the resulting damage becomes cytologically manifest only during mitosis, and that such damage may accumulate in successive cell divisions, as indicated by the presence of micronuclei of different ages, until the cell is in many cases no longer viable. Both the clinical effects which these substances are capable of producing, and the cytologic changes which form their basis, fully justify the description of such agents as "radiomimetic" (Dustin, 1947), even though there may be profound differences between them and ionizing radiations in the details of their action. The striking similarity in at least their biologic end results is also shown in other phenomena which can be induced by radiation and the mustards equally."

"Examples of this similarity are as follows: (1) local greying or bleaching of hair in mice, described by Hance and Murphy (1926; Hance, 1928) as produced by roentgen irradiation, and extensively studied by Chase and his co-workers (Chase, 1949, 1951; Chase, Quastler and Skaggs, 1947; Chase and Smith, 1949; Chase and Rauch, 1950), and for the case of chemical agents by Boyland and Sargent (1951); (2) characteristic acute and chronic degenerative changes in the bone-marrow and testis."

(emphasis ours)

The evidence so far obtained is insufficient to indict Benzethonium chloride as either a somatic mutagen or a radiomimetic drug. However, the observations made suggest strongly that it is cytotoxic and additional research in depth is indicated to ascertain that this widely used compound is as innocuous as has been hitherto assumed.

LEGENDS

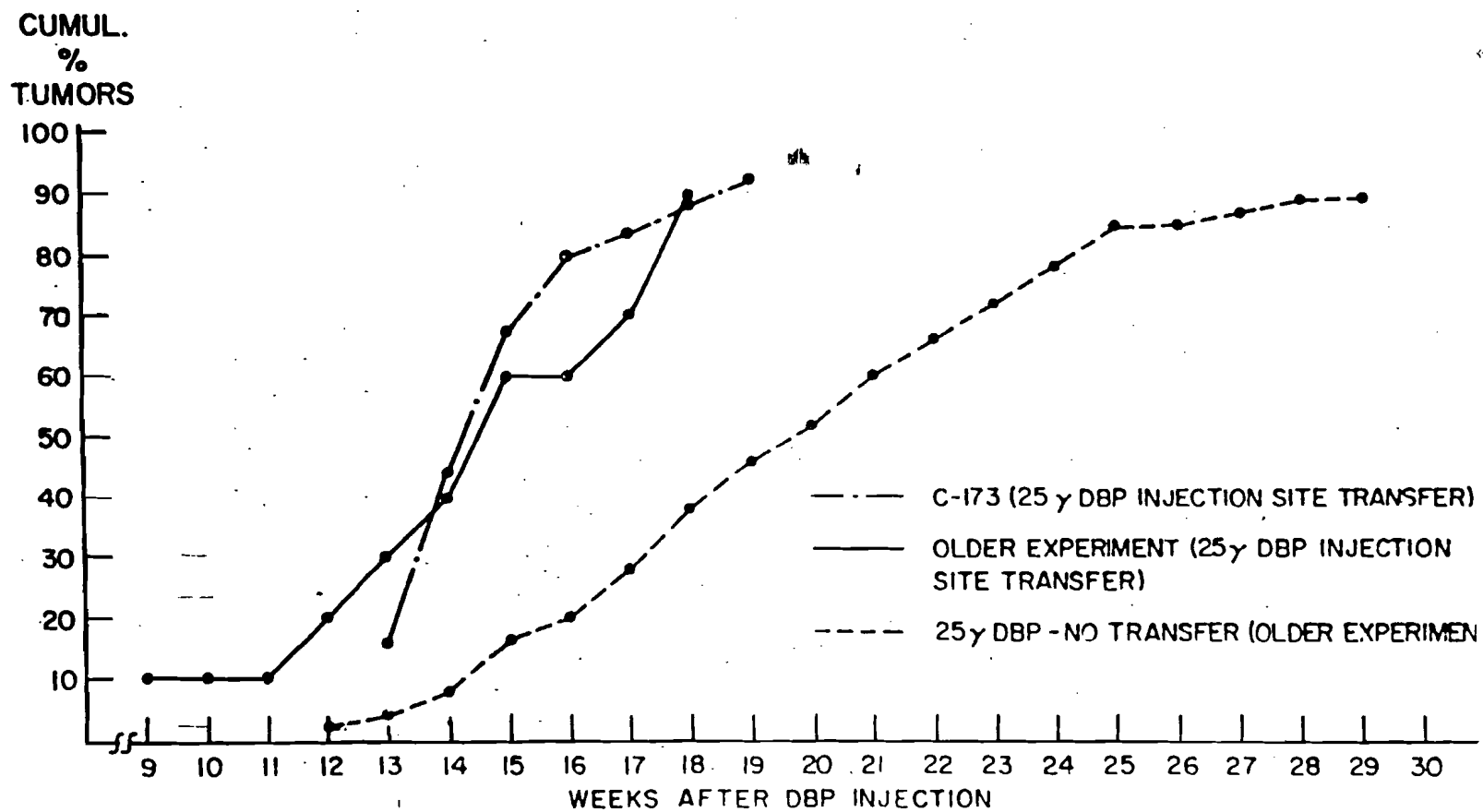
- Figure 1: Comparison of subcutaneous tumor induction (25γ DBP) by injection site transfer and without transfer in C57BL/6 mice.
- Figure 2: Hematoxylin-eosin-stained section of injection site transfer material. Original injection, 6 mg of pyridine in tricapylin 12/7/67; site transfer 1/9/68; sacrificed 5/21/68. Note foreign body reaction with round cell infiltration, giant cells, cholesterol clefts, cysts (magnification x 400).
(Path. No. 1303(5))
- Figure 3: Hematoxylin-eosin-stained section of an injection site transfer material from another animal in the same group (pyridine). This shows the same features with more emphasis on giant cells and granulomatous nature of reaction. Figures 2 and 3 are typical of all sections studied in this experiment (except the positive controls which showed fibrosarcomas similar to that shown in Figure 12).
(Path. No. 1303(3))
- Figure 4: Example of a Pathology Report.
- Figure 5: The following hematoxylin-eosin-stained sections (magnification x 200) show indistinguishable adenomas of the lung, such as seen in both A and CF 1 mice, observed in animals having received the treatments described. Illustrated in Figure 5, effects of 100γ DBP in an A/Jax mouse, sacrificed twenty weeks after injection. These lesions are identical with those observed in untreated animals and illustrated, for example, in H. Stewart's chapter, entitled Experimental Cancer of the Alimentary Tract, In The Physiopathology of Cancer, F. Homburger and W.H. Fishman, Eds., 1st edition, Hoeber-Harper, New York, pp. 3-45, 1953.
(Path. No. 1531)
- Figure 6: Effects of 100γ DBP in a CF 1 mouse, sacrificed twenty-three weeks after injection.
(Path. No. 1534)
- Figure 7: Effects of 500γ DBP in a CF 1 mouse, sacrificed twenty-three weeks after injection.
(Path. No. 1533(2))
- Figure 8: Effects of ethylene chlorohydrin in a CF 1 mouse, sacrificed twenty-eight weeks after first of seven injections.
(Path. No. 1536(2))
- Figure 9: Effects of Methyl Paraben in a CF 1 mouse, sacrificed twenty-eight weeks after first of seven injections.
(Path. No. 1538(2))

Figure 10: Effects of Benzethonium chloride in a CF 1 mouse, sacrificed
(Path. No. twenty-eight weeks after first of seven injections.
1539(3))

Figure 11: Effect of the seven compounds on subcutaneous tumor induction
with 12.5γ DBP in 0.1 cc tricaprylin.

Figure 12: Hematoxylin-eosin-stained section (magnification x 400). A
(Path. No. typical fibrosarcoma, invading muscle, as usually produced
930) by subcutaneous injection of DBP. The one illustrated here oc-
curred after injection of 500γ DBP followed by ethylene chloro-
hydrin.

Figure 1



COMPARISON OF SUBCUTANEOUS TUMOR INDUCTION (25γ DBP) BY
INJECTION SITE TRANSFER AND WITHOUT TRANSFER IN C57BL/6 MICE

Final Report - Project C-173
Figure 4

REQUEST FOR PATHOLOGY

(Check one)

BIO-RESEARCH INSTITUTE ()

BIO-RESEARCH CONSULTANTS (✓)

No. 68-1139

Tissue No. 8636-8641

Project No. C173

Request for: Histology ✓ Special Stain _____ Frozen Section _____

Cytology _____ Bacteriology _____ Gross Photo _____ Microphoto _____

Date of death/sacrifice 4-24-68 Animal No. 1-6 Sex ♂ Species M Strain C3280/6

Description of Material: Transfer site

History of Animal: Ethylate chlorohydrate (1.2mg/p.o. 1cc Tricoproline) inj 11-8-1967
late transfer 12-13-67. Sec'd 24 weeks after inj. of comp

What specific information is wanted from pathological study?

Formation of cancer cells?
other findings

REPORT

Requested by H. V. Rye

- 8636 #1 A piece of dense fibrous tissue throughout which are scattered many cysts of various sizes. In some areas some muscle is recognizable. There are also round cell infiltration and giant cells.
- 8637 #2 Same description as above. In addition, this section shows one area of a densely blue-staining granular material surrounding some giant cells, which could be a focus of calcification.
- 8638 #3 This shows the same tissue, but adjoining it a large area of necrosis with round cell and polymorphonuclear infiltration.
- 8639 #4 This shows the same tissue, but in this section is also contained a lymph node adjoining a granulomatous foreign-body reaction.
- 8640 #5 Fibrous tissue adjoining an area of fatty tissue which contains numerous small cysts.
- 8641 #6 This section shows a large cyst measuring 1/2 cm x 3 mm, containing an amorphous and, in places, reticulated pink-staining material surrounded by a thin fibrous capsule and embedded in fatty tissue.

Report by F. Homburger, M.D.

Date May 13, 1968

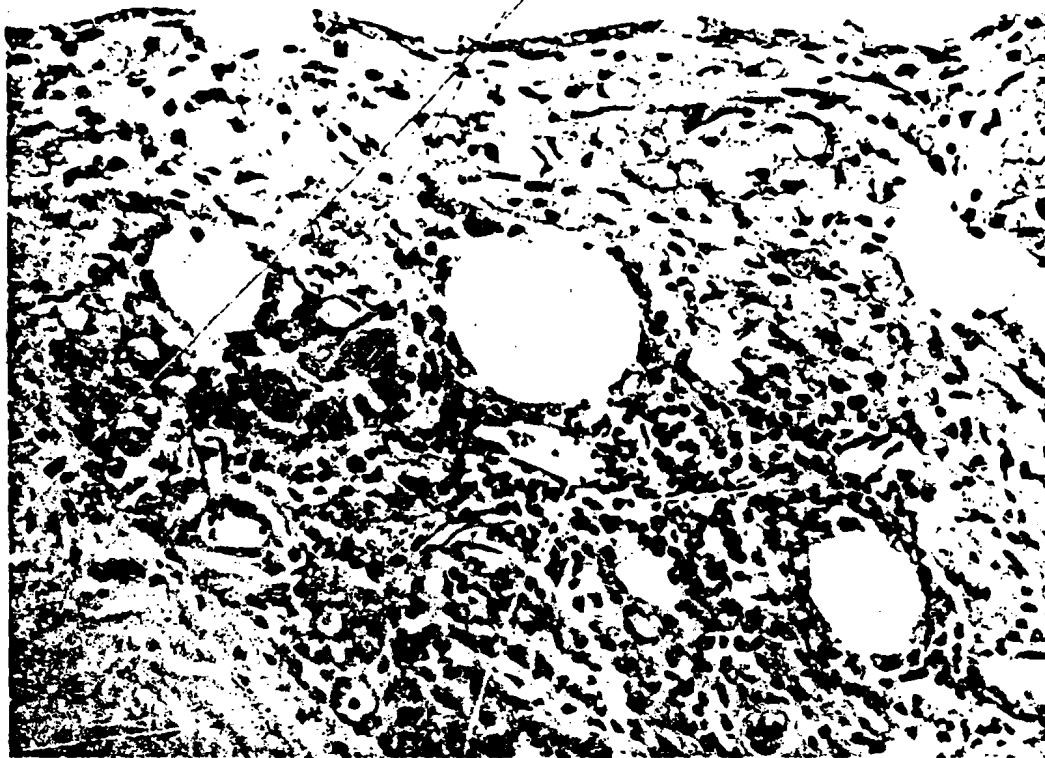


Figure 2



Figure 3

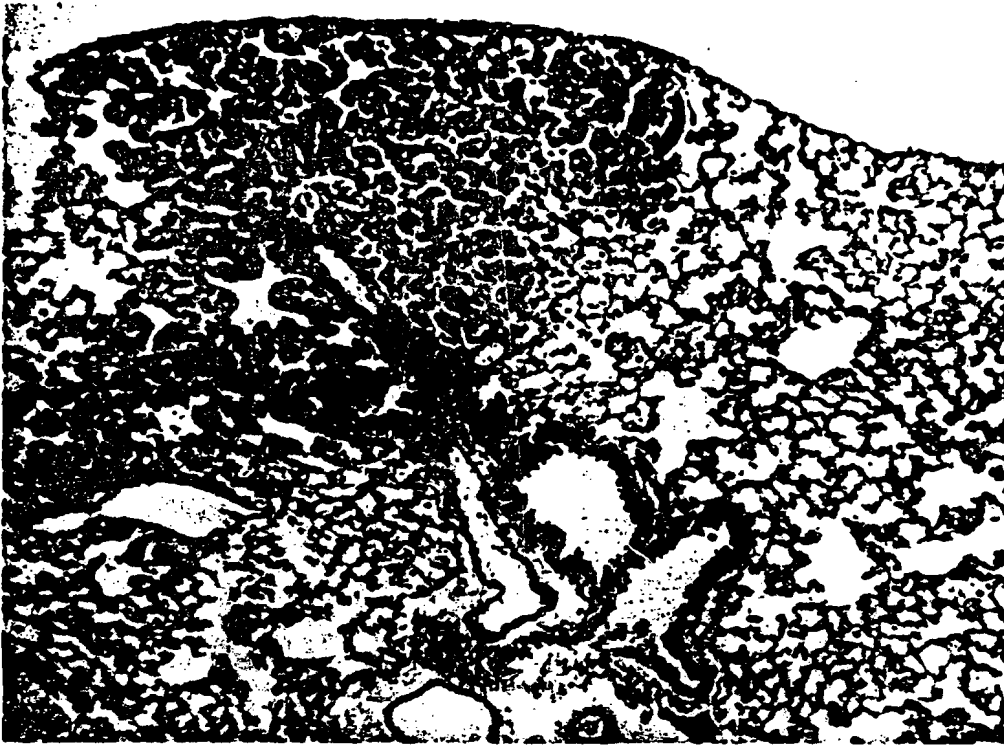


Figure 5

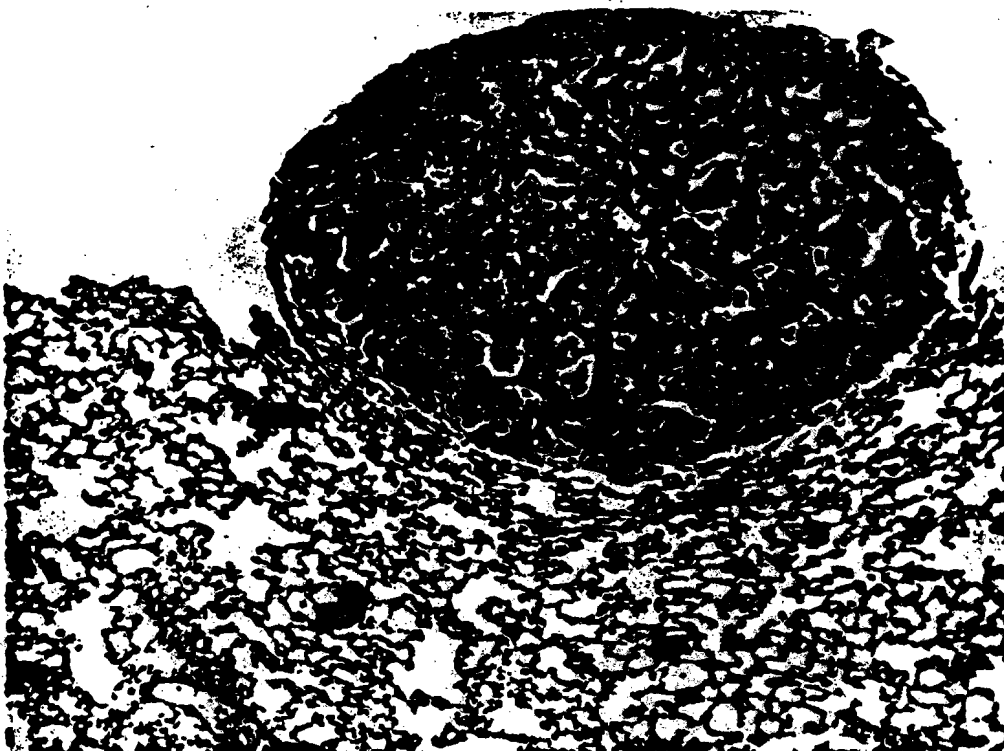


Figure 6

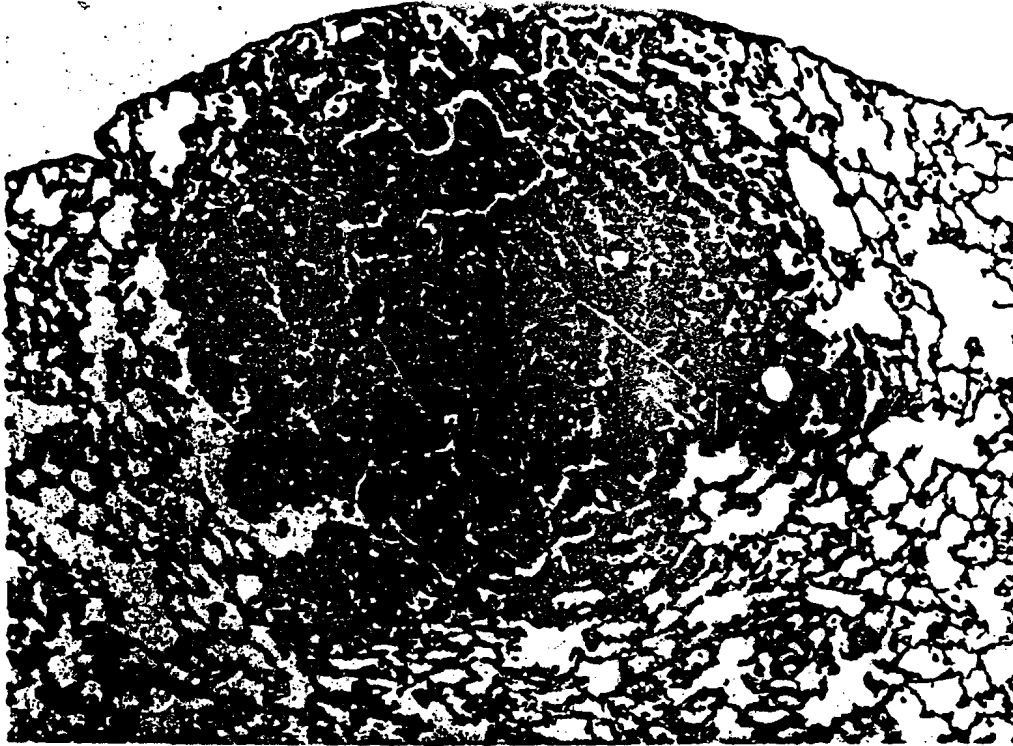


Figure 7

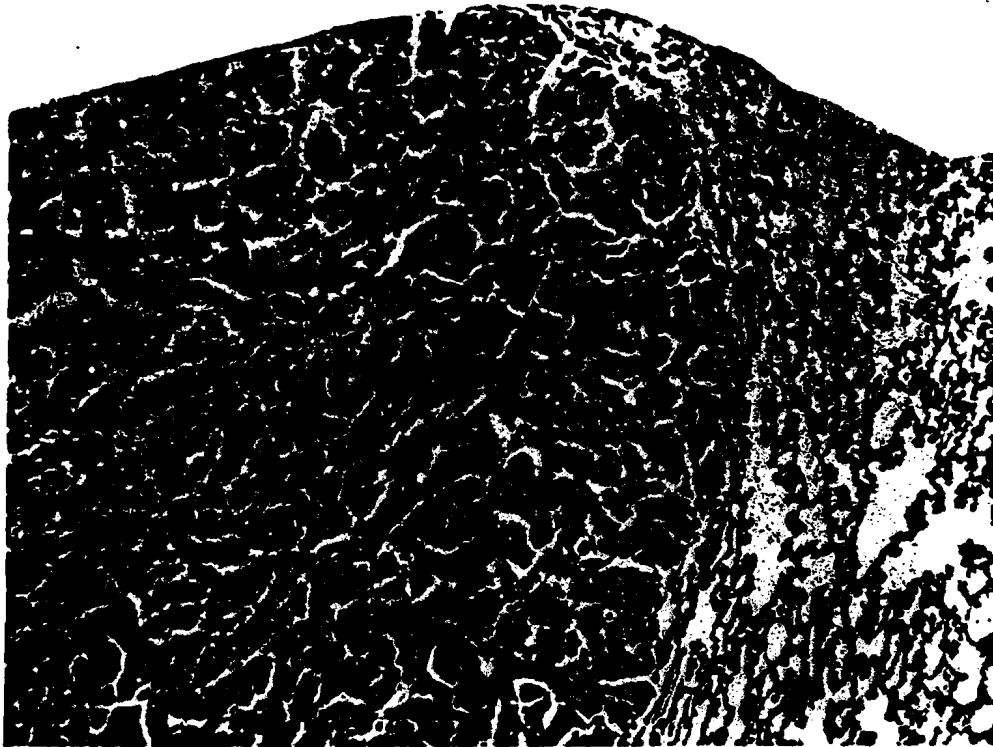


Figure 8



Figure 9

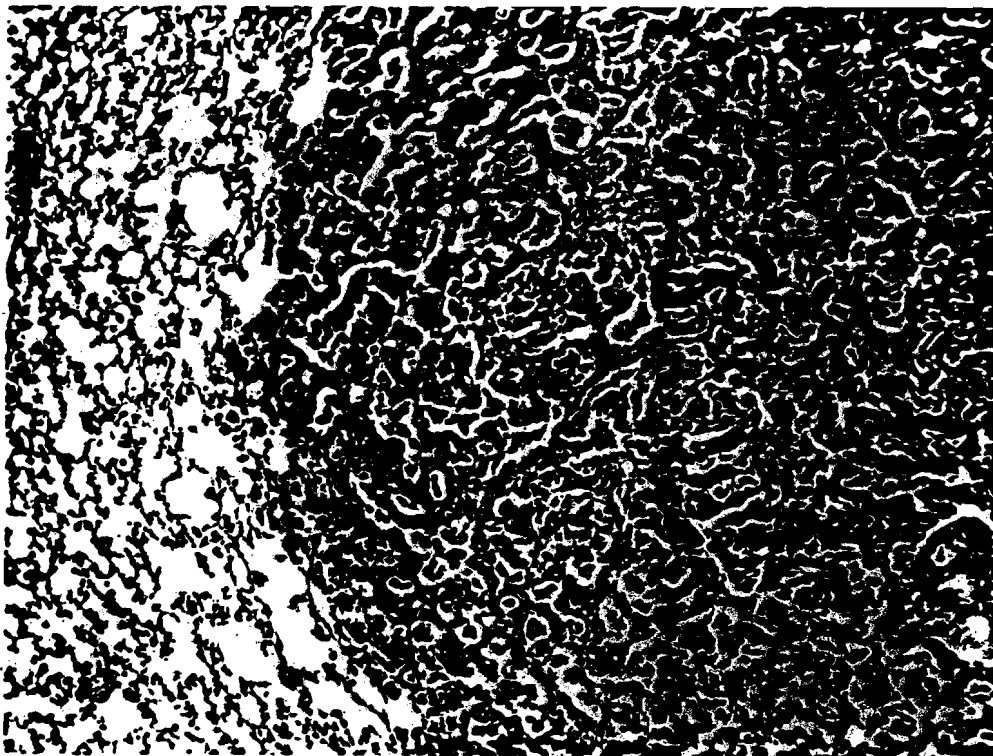
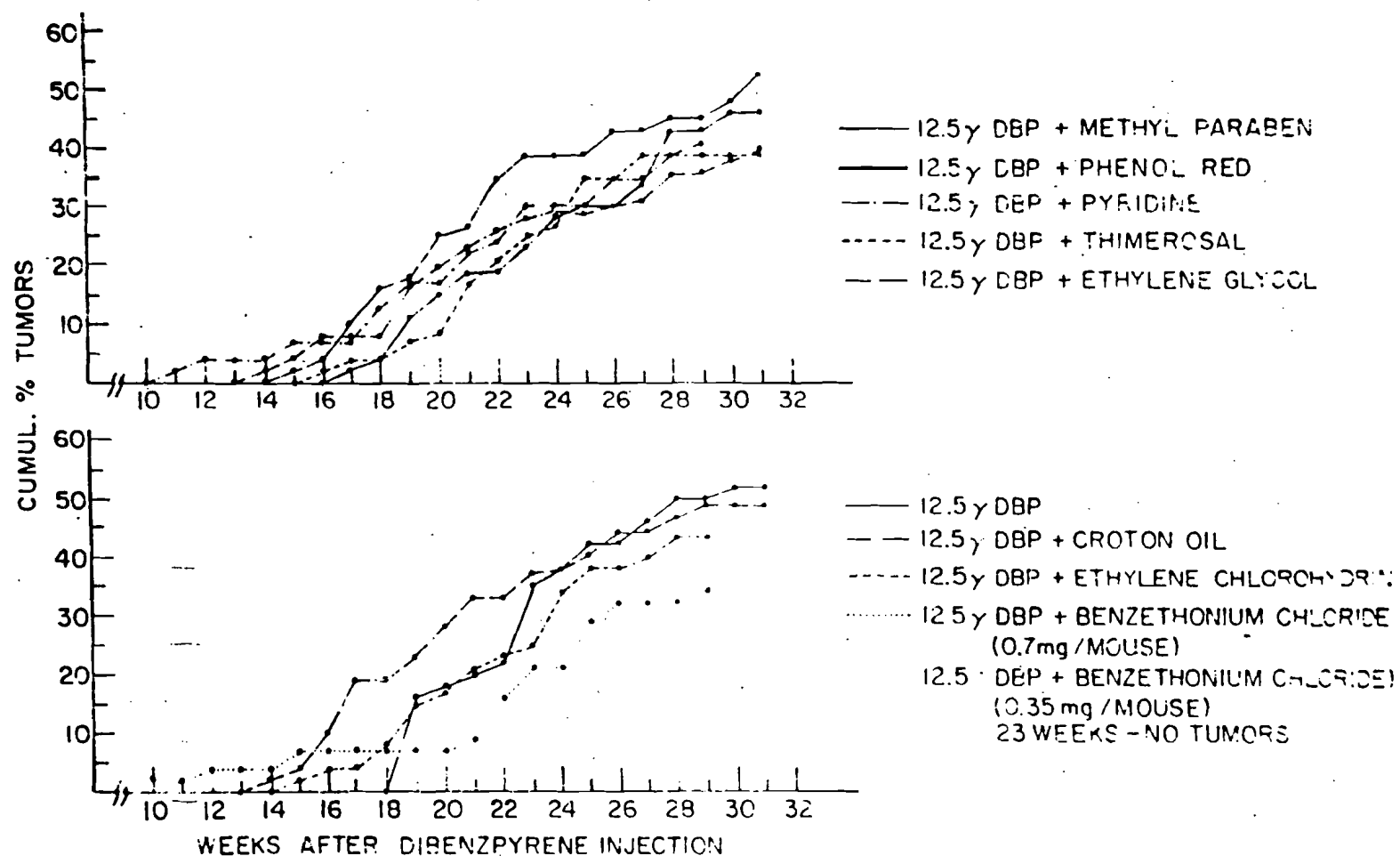


Figure 10

Figure 11



EFFECT OF THE SEVEN COMPOUNDS ON SUBCUT. TUMOR INDUCTION
WITH 12.5γ DBP IN 0.1cc TRICAPRYLIN



Figure 12

PB195153

FINAL REPORT

Contract No. PH 43-67-684

April 27, 1967 - April 26, 1969

**WARF Institute, Inc.
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Madison, Wisconsin**

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June 19, 1969

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16. Abstracts 684 random-bred Texas-Yale mice, less than 24 hours old were injected subcutaneously with either benzethonium chloride, ethylene chlorohydrin, ethylene glycol, methylparaben, phenol red, thimerosal or pyridine. The mice were examined after 15 months for evidence of carcinogenic activity. None of the compounds appeared to have any oncogenic potential in these mice.					
17. Key Words and Document Analysis. (a). Descriptors Carcinogenesis Benzethonium chloride Ethylene chlorohydrin Ethylene glycol Methylparaben Phenol red Thimerosal Pyridine					
17b. Identifiers/Open-Ended Terms					
17c. COSATI Field/Group					
18. Distribution Statement Anyone who requests			19. Security Class (This Report) UNCLASSIFIED		21. No. of Pages 139
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**Final Report - Abstract
WARF Institute, Inc.
Madison, Wisconsin**

**"The Injection of Newborn Mice with Seven Chemical
Adjuvants to Help Determine Their Safety in Use in Biologicals"**

A 15 month study employing the subcutaneous injection of newborn mice was initiated to determine possible carcinogenic properties of pyridine, ethylene glycol, 2-chlorethanol, benzethonium chloride, phenol red thimerosal, and methyl parasept. Saline was used as the vehicle and as the negative control. 1, 2, 5, 6-Dibenzanthracene (DBA) in both corn oil solution and saline suspension was used as the positive control. Two hundred males and 200 females composed the negative control group and 50 males and 50 females composed each level of the test and positive control groups. The test materials were given at 2 levels, a high level which allowed 50% survival of treated animals and a low level set at 10% of the high level. DBA was given at 2 levels chosen from literature and known to have produced a high and low level of tumor incidence.

The test substances were administered subcutaneously in mice less than 24 hours old by inserting a 27 gauge needle at the base of the tail and depositing 0.05 ml of solution in the area of the neck and scapula.

Animals were observed daily and body weights recorded weekly for 13 weeks and every other week thereafter. Average individual body weights are reported weekly for each sex in each treatment.

At approximately 15 weeks of age the mouse population was afflicted with a respiratory condition which produced a moderate level of mortality.

Although mortality was higher than would be preferred during this early period, it was decreased and maintained at a low level through the majority of the test period allowing adequate survival at termination for gross and histologic examination. Animals sacrificed at termination showed a comparatively low level of serious non-neoplastic pathology.

Tumor incidence at the site of injection was as anticipated in the positive control groups receiving levels of DBA in oil. DBA in saline did not produce similar tumors at the site of injection, but the high level injection did result in a significant increase in number of lung adenomas. Other tumor occurrence presented a picture of random distribution among control and test groups typical of spontaneous tumors in mice. There was no correlation between a test material and/or dose level and neoplasm production.

Under the conditions of this study, in mice shown to be susceptible by their response to the known carcinogen DBA, the compounds tested were shown to not be chemical carcinogens.

Final Report
WARF Institute, Inc., Madison, Wisconsin

Objective of study:

To confirm that the compounds tested are safe as used in biological products, with special emphasis on determining any possible carcinogenicity. Compounds being tested are:

Pyridine - Fisher Lot 762678

Ethylene glycol - Fisher Lot 764959

2-Chlorethanol - Eastman Cpd. 131

Benzethonium chloride - Rohm & Haas Lot 9781

Phenol Red - National Aniline Lot 2606P

Thimerosal, N.F. - Lilly Control OSE40C

Methyl Parasept - Heyden Lot 1316

General description of study:

The procedure employed generally followed that described by Kelly, M.G. and O'Gara, R.W., Induction of Tumors in Newborn Mice with Dibenz(a,h) Anthracene and 3-methylcholanthrene, J. Nat. Cancer Inst. 26:651-679 (1961), and involved the administration of a single dose of the test material subcutaneously in newborn mice within 24 hours after birth, followed by observation for 15 months. The dosage levels chosen were that level causing approximately 50% mortality of treated animals as the high level and one-tenth that level for the low level. Sterile saline was used as the diluent for the test materials and as the negative control material. 1, 2, 5, 6-Dibenzanthracene (DBA) was used as the positive control and administered at 2 levels, one high and one low based on literature

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reports of level of activity and in two different vehicles, sterile saline and corn oil. The following general group allotment was used:

Table 1

Negative control
Test material (high level)
Test material (low level)
Positive control in corn oil (high level)
Positive control in corn oil (low level)
Positive control in saline (high level)
Positive control in saline (low level)

A preliminary series of injections was conducted to determine the high dosage level or dosage resulting in 50% mortality of treated animals. This study resulted in the selection of dosage levels and the injection schedule noted in Table 2.

Method

Texas-Yale albino mice, Texas Inbred Mice Co., Houston, Texas, were used in this study. The mice were maintained on Purina Breeder Chow from five weeks of age through litter production and weaning. The mice were housed in double screen bottom (16 x 10 x 7 inches) cages, 10 mice per cage, until breeding began when three females and one male were placed in single screen bottom cages. When pregnancy was obvious, the female was removed to a 11.5 x 7.5 x 5 plastic shoe box type cage bedded with ground treated corn cob*, where she remained until her litter was weaned.

*San-I-Cel (Paxton Processing, Inc., Paxton, Illinois).

Each morning the cages were checked for newborn litters and any young were weighed and injected subcutaneously with the appropriate compound. At birth litters were assigned at random to the groups. To inject solutions a 27 gauge needle was inserted under the skin at the base of the tail and inserted along the hypodermal layer to the area of the neck and shoulders where .05 ml of the compound dilution was deposited. Minimal leakage was observed with this method. Plastic disposable gloves were worn when handling the animals to avoid rejection of the litter by the female. 0.25 cc glass syringes were used for the test. After injecting a litter, the syringe and needle were washed thoroughly in hot soapy water, rinsed in alcohol, individually wrapped in a clean white paper towel and autoclaved at 248°F for 20 minutes.

At weaning the animals were sexed, ear marked and placed in screen bottom cages, 5 animals per cage. Commercial laboratory pellets and water were supplied ad libitum. The animals were weighed weekly for the first thirteen weeks and every other week for the remainder of the test.

When mice were approximately 15 weeks of age a number of animals developed symptoms of respiratory disease which appeared to be progressing. Cages of animals showing evidence of illness or weight loss were transferred to shoe box cages and isolated from the main group of animals. Approximately 500 animals were isolated although a portion of these showed minimal symptoms such as slight weight loss. Some mortality occurred in isolated animals. This procedure appeared to

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halt the immediate spread of the condition in the main population. After several weeks of isolation it was apparent that the main population contained a significant number of new cases indicating a continuing problem. Transfer of groups or individuals showing symptoms was continued. All mortalities were examined grossly and tissues collected according to the schedule in the protocol. During this same period it was found that the mice isolated in the shoe box cages were developing clinical infestations of sarcoptic mange mites. Examining the general population, a low level infestation was discovered in the entire group. The entire population was treated with a DDT dust preparation at 15 and 19 weeks. Whether the mites were introduced in bedding or on the parent animals was not determined.

Mice from other studies at the laboratory were examined. Although from a different source, they had been housed in adjacent rooms and these mice at 15 months, did not show the respiratory problems or mite infestation mentioned above.

Results and Discussion

Body weight and survival data are presented on a weekly basis in Tables 5 through 14 and are summarized in Table 3A. The summary, Table 3A, presents data at termination. When weighings were put on a bi-weekly basis, portions of each group are generally weighed every week, but in ethylene glycol group all the animals in a group are weighed at the same week and therefore body weight data is noted every other week. Table 3B summarizes the incidence and location of all non-neoplastic lesions observed grossly or histologically in animals dying during test. Table 3C presents similar data for animals sacrificed terminally.

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for individual animals in tables 15 through 52 and summarized on table 4A. Similar data on animals sacrificed at termination are presented in summary on table 4B and individual in tables 53 through 90. On the pages facing table 15 and table 53 is an explanation of the table arrangement and data presented in the tables that follow.

Body weight data in general show normal average animal weights and the variation is generally within limits expected. In groups receiving Thimerosal, males and females on the high level dose, had notably lower body weights when compared with the animals on the low level dose. In female groups receiving Benzethonium chloride, methyl parasept and DAB (in corn oil), females receiving the higher dose level showed somewhat lower body weights. The weights generally bracket the weight noted for the negative control females.

Survival data reveals no clear-cut pattern with respect to dose levels, although there is some correlation of higher mortality with higher dose in groups receiving benzethonium chloride and DBA. Mortality differences between groups does not appear to correlate with test material administered. Males appear to be more susceptible than females to the infection. A moderate mortality has occurred throughout the period from the first appearance of the respiratory condition. During the last 9 months previous to termination, mortality was quite constant and averaged approximately 2 to 3 animals per day of the approximately 1500 surviving animals.

Continuing close attention to the sanitation and general husbandry of these animals limited the mortality. Continual observation for mor-

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talities and sacrifice of moribund animals has allowed collection of an adequate tissue sampling. During the terminal months of the study, these procedures were emphasized. Very few animals in the total study were cannibalized or otherwise lost so we were unable to conduct at least a gross examination for tumors.

Tables 3B and 3C indicate the general level of non-neoplastic pathology. The lung lesions noted are those of severe chronic respiratory disease. Although this was the frequent lesion of those animals dying on test and was, in many instances the prime cause of mortality, the animals surviving to termination showed a remarkably low percent of respiratory tract lesions.

Kidney lesions were noted frequently on histologic examination but were predominantly minimal to slight dilation of tubules with some cystic changes. This occurred in control as well as test animals, not apparently related to treatment or dose level and was considered a spontaneous lesion of the aging mice.

Other urinary tract lesions were of primarily two types and were sex related. Males showed a typical infection of the lower urinary tract which, in some cases, resulted in ascending infection of partial urethral blockage which caused pathologic changes in the urinary bladder and kidney pelvis. In the females, nearly every individual showed some degree of cystic endometritis. This varied from minimal lesions seen only histologically to severe enlargement which was obvious grossly. The urinary tract lesions noted appear to be normal spontaneous lesions of aging in this mouse population.

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control and test groups and not related to treatment or dose level. Except for the high incidence of respiratory disease which we have not encountered in any other studies to date, the level of non-neoplastic lesions were not unusual as far as experience with random bred Swiss white type mice in long term studies at this laboratory. Even the level of respiratory tract lesions was low in those animals surviving to termination.

Tumor incidence as summarized in tables 4A and B show 3 major tumor types occurring during the study: lung adenoma, lymphosarcoma, and sarcoma of fibro and/or spindle-cell type. Other neoplasm types observed, were infrequent and randomly scattered in control and test groups.

Lung adenomas were observed in most groups and primarily in those animals which survived to termination of the study. Except in the groups receiving the positive control material (DBA), the lung adenomas were noted randomly distributed among test and negative control animals with no correlation to particular test materials and no dose related response. In the groups receiving DBA in saline there is a significant increase in number of animals with lung adenoma and this is directly related to the dosage level. In animals receiving DBA in oil, there is some slight indication of similar response, but apparently susceptible animals had already been eliminated by sarcomas or the material has a variation in site of activity depending on vehicle and form in which

it is given. It also is apparent in this study that lung adenomas did not develop to any great extent until the 12 to 15 month period.

The incidence of lymphosarcomas observed was typical of the frequency we have noted in mice in previous studies, and there is no apparent correlation between the lymphosarcoma and treatments or dosage levels.

The other sarcomas noted were primarily in two locations, the injection site in the case of animals receiving DBA in oil, and the uterus in females of a majority of the groups. The sarcomas at the injection site were limited to the positive control groups and typical of the type produced by DBA. The tumors noted in the uteri were typically fibrosarcomas, randomly distributed among the various groups and generally associated with chronic endometritis. Other types and locations of sarcomas were infrequent and not related to treatment, sex or dose level.

It is interesting to note, the development of spontaneous mammary tumors was absent in this strain of mice.

Conclusion

In a study involving single injection of day-old mice with seven compounds and subsequent observation for 15 months, there were no indications of chemical carcinogenicity produced, by any of the test compounds. 1, 2, 5, 6-Dibenzanthracene (DBA) produced the anticipated carcinogenic response at both high and low levels when injected in corn

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oil solution producing sarcoma growth at the site of injection. DBA injected in a saline suspension did not produce neoplasms at the site of injection, but produced a significant increase in the incidence of lung adenoma in the animals receiving only the high dose level of DBA.

Signed _____

By and for WARF INSTITUTE, INC.

Date June 20, 1969

Table 2

Resume of Mouse Injections

Compound	Dilution**	Dose (mgm)	Number of mice			% mortality
			Injected	Dead	Surviving	
Saline	--	(0.05 ml)	532	108	424	20
Pyridine	1 to 37.5	1.33	267	152	115	57
	1 to 375	0.133	185	74	111	40
Ethylene glycol	1 to 5	10.0	242	123	119	51
	1 to 50	1.0	161	43	118	27
2-Chlorethanol	1 to 300	0.17	294	168	126	57
	1 to 3000	0.017	159	48	111	30
Benzethonium	1 to 1500	0.034	203	84	119	41
	1 to 15,000	0.0034	135	18	117	13
Phenol Red***	1 to 30	1.7	144	26	118	18
	1 to 300	0.17	165	47	118	28
Thimerosal $\frac{1}{8}$	1 to 625	0.08	209	89	120	43
	1 to 6250	0.008	149	31	118	21
Methyl Parasept	1 to 100	0.5	136	23	113	17
	1 to 1000	0.05	162	46	116	28
DBA* (in oil)	1 to 7462	0.0067	153	27	126	18
	1 to 250,000	0.0002	153	38	115	24
DBA* (in saline)	1 to 7462	0.0067	169	43	126	25
	1 to 250,000	0.0002	153	51	102	33

*Dosage determined from levels reported in literature.

**All dilutions in saline except for 2 DBA groups in oil.

0.05 cc of dilution given to each mouse.

***Unable to produce 50% mortality. Used near saturated solution of test material in saline as high dose level.

Table 3
Body Weight and Survival Summary

Group	Sex	Dilution	animals allotted	(gms)		Percent of animals surviving	mortality to date
				Weaning	Terminal		
Saline	M	--	200	10.8	35	47.5	105
Saline	F	--	2200	10.7	31	69.0	62
Pyridine	M	1-37.5	50	10.6	33	58	21
Pyridine	F	1-37.5	50	10.2	30	78	11
Pyridine	M	1-375	50	10.2	35	48	26
Pyridine	F	1-375	50	10.0	31	62	19
Ethylene glycol	M	1-5	50	9.9	36	54	23
Ethylene glycol	F	1-5	47	9.4	28	64	17
Ethylene glycol	M	1-50	50	10.2	36	60	20
Ethylene glycol	F	1-50	50	9.9	30	72	14
2-Chlorethynol	M	1-300	50	12.5	35	56	22
2-Chlorethynol	F	1-300	50	11.3	32	88	6
2-Chlorethynol	M	1-3000	47	9.6	35	49	27
2-Chlorethynol	F	1-3000	50	11.9	32	78	11
Benzethonium	M	1-1500	50	10.9	34	40	30
chloride	F	1-1500	50	9.8	30	50	25
"	M	1-15,000	50	10.6	34	44	28
"	F	1-15,000	50	10.1	32	72	14
Phenol Red	M	1-30	50	10.6	34	52	24
Phenol Red	F	1-30	50	10.1	33	76	12
Phenol Red	M	1-300	50	9.9	35	36	32
Phenol Red	F	1-300	50	10.0	33	70	15
Thimerosal	M	1-625	50	10.6	31	60	20
Thimerosal	F	1-625	50	11.0	29	78	11
Thimerosal	M	1-6250	50	9.6	35	60	20
Thimerosal	F	1-6250	50	9.9	33	66	17
Methyl parasept	M	1-100	50	10.3	35	64	18
Methyl parasept	F	1-100	50	10.5	30	62	19
Methyl parasept	M	1-1000	50	10.0	35	48	26
Methyl parasept	F	1-1000	50	10.4	32	76	12
DBA-corn oil	M	1-7462	50	10.3	36	32	34
DBA-corn oil	F	1-7462	50	10.3	29	44	28
DBA-corn oil	M	1-250,000	50	10.5	36	46	27
DBA-corn oil	F	1-250,000	50	10.2	32	58	21
DBA-saline	M	.75 mg/ml	50	9.8	34	40	30
DBA-saline	F	.75 mg/ml	45	10.0	30	66	15
DBA-saline	M	.075 mg/ml	50	11.2	35	54	23
DBA-saline	F	.075 mg/ml	48	10.3	31	81	9

*DBA-011 mortality reflects animals sacrificed because of tumors.

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Table 3B
Incidence of non-neoplastic pathology
Animals dead on test, number and location

Males

<u>Treatment</u>	<u>Lung</u>	<u>Kidney</u>	<u>Spleen</u>	<u>Lymph node</u>	<u>Adrenal</u>	<u>Urogenital tract</u>	<u>Other</u>
Saline	7	4	0	0	0	22	3
Pyridine Lo	5	0	0	0	0	6	1
" Hi	0	0	0	0	0	2	0
Eth. glycol Lo	0	1	0	0	0	4	0
" Hi	0	3	0	0	0	4	0
Chlorethanol Lo	4	3	0	0	0	5	1
" Hi	2	0	0	0	0	1	1
Benzethonium Lo	2	5	2	1	0	7	1
" Hi	2	1	0	0	0	1	0
Phenol Red Lo	3	4	0	0	0	9	0
" Hi	1	0	0	0	0	1	1
Thimerosal Lo	3	2	1	0	0	3	3
" Hi	1	0	0	0	0	1	1
Meth. parasept Lo	3	2	1	0	0	11	0
" Hi	6	1	0	0	0	5	1
DBA-oil Lo	1	4	0	0	0	2	1
" Hi	2	3	0	0	0	6	1
DBA-saline Lo	3	3	0	0	0	7	1
" Hi	3	1	0	0	0	3	0

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Table 3C

Animals dead on test, number and location

Females

<u>Treatment</u>	<u>Lung</u>	<u>Kidney</u>	<u>Spleen</u>	<u>Lymph node</u>	<u>Adrenal</u>	<u>Urogenital tract</u>	<u>Other</u>
Saline	13	3	1	0	0	3	3
Pyridine Lo	7	2	0	0	0	0	0
" Hi	0	0	0	0	0	1	0
Eth. glycol Lo	4	1	0	0	0	0	0
" Hi	5	1	0	0	0	0	0
Chlorethanol Lo	1	0	0	0	0	0	0
" Hi	2	0	0	0	0	0	2
Benzethonium Lo	0	0	0	0	0	0	0
" Hi	1	0	0	0	0	0	1
Phenol Red Lo	0	0	0	0	0	0	0
" Hi	3	0	0	0	0	1	1
Thimerosal Lo	4	1	0	0	0	0	2
" Hi	1	0	0	0	0	0	0
Meth. parasept Lo	3	0	0	0	0	1	1
Hi	2	1	0	0	0	0	0
DBA-oil Lo	1	0	0	0	0	0	3
" Hi	1	0	0	0	0	0	2
DBA-saline Lo	2	0	0	0	0	0	0
" Hi	1	0	0	0	0	0	1

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Table 4A
Summary of Tumor Incidence
Animals dead on test

Males

Treatment and Table Number	Benign neoplasms				Malignant neoplasms			Total animals	
	Lung ade- noma	Adenoma or fibroma	Skin papil- loma	Heman- gioma	Sarcoma		Carcin- oma	With tumors	Exam. gross
					Lympho.	Other			
Saline-15	-	-	-	-	-	-	-	0	94
Pyridine	-	-	-	-	-	-	-	-	-
Lo-17	-	-	-	-	1	-	-	1	26
Hi-19	-	1	-	-	1	-	-	0	20
Eth. glycol	-	-	-	-	-	-	-	-	-
Lo-21	-	-	-	-	-	-	-	0	19
Hi-23	-	-	-	-	-	-	-	0	23
Chlorethanol	-	-	-	-	-	-	-	-	-
Lo-25	-	-	-	-	2	-	-	2	23
Hi-27	1	-	-	-	-	-	-	1	20
Benzetho- nium	-	-	-	-	-	-	-	-	-
Lo-29	-	-	-	-	-	-	-	0	24
Hi-31	-	-	-	-	1	-	-	1	29
Phenol red	-	-	-	-	-	-	-	-	-
Lo-33	-	-	-	-	-	-	-	0	32
Hi-35	-	-	-	-	-	-	-	0	21
Thimerosal	-	-	-	-	-	-	-	-	-
Lo-37	-	-	-	-	2	-	-	2	20
39	-	-	-	-	-	-	-	0	20
Me.n. para- sept	-	-	-	-	-	-	-	-	-
Lo-41	1	-	-	-	-	-	-	1	24
Hi-43	1	-	2	-	-	-	-	3	18
DBA-oil	-	-	-	-	-	-	-	-	-
Lo-45	-	-	-	-	1	5	-	6	26
Hi-47	-	-	1	-	-	15	-	16	36
DBA-saline	-	-	-	-	-	-	-	-	-
Lo-49	-	1	2	-	2	-	-	4	30
Hi-51	1	-	-	-	1	-	-	2	23

Table 4A (continued)
Summary of Tumor Incidence

Females

Treatment and Table Number	Benign neoplasms				Malignant neoplasms			Total animals	
	Lung adenoma	Adenoma or fibroma	Skin papil- loma	Heman- gioma	Sarcoma		Carcin- oma	With tumors	Exam. gross
					Lympho.	Other			
Saline-15	1	-	-	-	2	-	1	3	50
Pyridine	-	-	-	-	-	-	-	0	19
Lo-18	-	-	-	-	1	-	-	1	12
Hi-20	-	-	-	-	-	-	-	-	-
Eth. glycol	-	-	-	1	-	-	-	1	14
Lo-22	-	-	-	-	1	-	1	2	17
Hi-24	-	-	-	1	1	-	-	2	8
Chlorethanol	-	-	-	-	1	-	-	1	5
Lo-26	-	-	-	1	-	-	-	-	-
Hi-28	-	-	-	-	-	-	-	-	-
Benzetho- nium	-	-	-	1	-	-	-	1	15
Lo-30	-	-	-	-	-	-	-	0	25
Hi-32	-	-	-	-	-	-	-	-	-
Phenol red	-	-	-	-	-	-	-	0	11
Lo-34	-	-	-	-	1	1	-	2	13
Hi-36	-	-	-	-	-	-	-	-	-
Thimerosal	-	-	-	-	-	-	-	0	17
Lo-38	-	-	-	-	-	1	-	1	12
Hi-40	-	-	-	-	-	-	-	-	-
N. a. para- sept	-	-	-	-	-	-	-	-	7
Lo-42	-	-	-	-	-	1	-	1	19
Hi-44	-	-	-	-	-	-	-	-	-
DBA-oil	-	-	-	-	1	5	-	6	21
Lo-46	-	1	-	-	1	11	-	13	26
Hi-48	-	-	-	-	-	1	-	1	15
DBA-saline	-	-	-	-	-	-	-	0	7
Lo-50	-	-	-	-	-	-	-	-	-
Hi-52	-	-	-	-	-	-	-	-	-

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Table 4B
Summary of Tumor Incidence
Animals killed at termination
Males

Treatment and Table Number	Benign neoplasms				Malignant neoplasms			Total animals	
	Lung adenoma	Adenoma or fibroma	Skin papilloma	Hemangioma	Sarcoma		Carcinoma	With tumors	Exam. histo.
					Lympho.	Other			
Saline-53	1	1	1	-	1	-	-	4	33
Pyridine									
Lo-55	1	-	-	-	-	-	-	1	24
Hi-57	2	-	-	-	1	-	-	3	26
Eth. glycol									
Lo-59	-	-	-	-	-	-	-	0	22
Hi-61	1	-	-	-	1	-	-	2	22
Chlorethanol									
Lo-63	1	-	-	-	-	-	-	1	16
Hi-65	-	-	-	-	-	-	-	0	22
Benzethonium									
Lo-67	1	-	-	-	-	-	-	1	17
Hi-69	-	-	-	-	-	-	-	0	19
Phenol Red									
Lo-71	-	-	-	-	-	-	-	0	15
Hi-73	1	-	1	-	-	1	-	3	25
Thimerosal									
Lo-75	1	-	-	-	-	-	-	1	20
Hi-77	1	-	1	-	-	-	-	2	25
M. para-									
sc.									
Lo-79	3	-	-	-	-	-	-	3	20
Hi-81	-	-	-	-	-	-	-	0	20
DBA-oil									
Lo-83	1	-	-	-	1	1	-	2	19
Hi-85	2	1	-	-	-	1	-	3	13
DBA-saline									
Lo-87	-	-	-	-	1	-	-	1	18
Hi-89	10	-	-	-	1	1	-	12	24

Table 4B.(continued)
Summary of Tumor Incidence

Treatment and Table Number	Benign neoplasms				Malignant neoplasms			Total animals	
	Lung adenoma	Adenoma or fibroma	Skin papilloma	Hemangioma	Sarcoma		Carcinoma	With tumors	Exam. histo.
					Lympho.	Other			
Saline-54	9	1	-	1	7	1	-	13	43
Cyridine									
Lo-56	-	-	-	2	4	2	-	7	25
Hi-58	-	1	-	-	5	2	-	7	26
Eth. glycol									
Lo-60	-	-	-	-	5	1	-	5	21
Hi-62	-	-	-	-	2	2	-	4	23
Chlorethanol									
Lo-64	-	-	-	4	2	1	-	11	25
Hi-66	3	-	-	1	7	-	-	11	24
Benzethonium									
Lo-68	1	-	-	-	3	-	-	4	27
Hi-70	2	-	-	-	1	-	-	3	22
Phenol red									
Lo-72	1	-	-	-	1	-	-	2	28
Hi-74	1	-	-	-	5	2	-	7	28
Thimerosal									
Lo-76	2	-	-	-	1	-	-	3	23
Hi-78	3	-	-	1	1	1	-	6	23
M. para-									
Lo-80	-	1	-	-	4	-	-	4	24
Hi-82	2	-	-	-	-	-	-	2	20
DBA-oil									
Lo-84	1	-	-	-	1	2	-	4	24
Hi-86	4	-	-	-	1	1	-	5	21
DBA-saline									
Lo-88	2	1	-	-	1	-	-	4	21
Hi-90	13	-	-	-	6	1	-	18	30

Table 5. Body Weight and Survival Data

Week	Saline			
	Body weight - gms (average individual)		Survival (%)	
	Male	Female	Male	Female
1	16	15	100	100
2	21	19	100	100
3	23	21	100	100
4	25	22	100	100
5	27	23	100	100
6	28	24	100	99
7	28	25	100	99
8	29	25	99	99
9	29	25	98.5	99
10	30	25	98.5	99
11	30	25	98.5	98
12	31	26	97.5	97.5
13	31	26	96.5	97.5
14	31	26	94.5	97.5
15	32	26	91.5	94.5
16	31	27	89.5	93.0
17	31	27	88.5	93.0
18	32	27	87.5	92.5
19	32	27	86.5	92.5
20	32	28	84.5	91.0
21	31	28	94.0	89.5
22	32	28	83.0	88.0
23	33	29	82.5	87.5

Table 5. Body weight and survival data

<u>Week</u>	<u>Body weight - gms</u> <u>(average individual)</u>		<u>Survival - %</u>	
	<u>Male</u>	<u>Female</u>	<u>Male</u>	<u>Female</u>
24	32	28	82.5	87.0
25	32	30	82.5	87.0
26	31	28	82.0	87.0
27	33	29	82.0	86.5
28	32	29	81.5	86.5
29	33	29	79.5	86.5
30	32	28	78.0	86.0
31	32	29	76.5	86.0
32	32	29	76.5	86.0
33	33	29	76.0	85.0
34	33	29	75.0	84.5
35	34	29	73.0	84.5
36	33	30	72	82.5
37	34	30	71	82.5
38	34	29	71.	81.5
39	34	29	68.5	81.5
40	34	30	68.5	81.5
41	34	30	68.5	81.5
42	34	30	68.5	81.
43	34	30	67.5	81
44	34	31	67.5	80.5
45	35	31	65	80.5
46	35	31	64.5	80.

Table 5. Body Weight and Survival Data

Week	Saline			
	Body weight (gms) (average individual)		Survival (%)	
	Male	Female	Male	Female
47	34	31	63	79.5
48	34	31	63	79.
49	35	32	62	78.5
50	34	31	60	78
51	36	31	59	78
52	35	31	57	77.5
53	35	31	56	76
54	35	31	56.5	75
55	35	31	54	74.5
56	35	31	53	72.5
57	35	31	52	72.5
58	35	31	50.5	71.5
59	34	31	50.5	70.5
60	35	31	49	69

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Table 6. Body weight and Survival Data

Pyridine

Body weight (gms)

ek	Male		Female		Male		Female	
	Low	High	Low	High	Low	High	Low	High
1	16	16	15	15	100	100	100	100
2	21	21	18	19	100	100	100	100
3	24	24	20	20	100	100	100	100
4	26	25	21	21	100	100	100	100
5	27	27	22	22	100	100	100	100
6	27	28	23	24	100	100	100	100
7	28	29	24	24	98	100	100	100
8	29	30	25	26	98	100	100	100
9	30	29	26	26	98	100	100	100
10	31	30	26	26	96	100	100	100
11	31	30	27	26	96	96	100	100
12	32	30	26	26	94	96	100	100
13	32	30	26	27	90	92	100	100
14	32	32	26	27	90	92	100	100
15	32	31	27	28	90	90	96	100
16	32	31	28	27	90	90	96	100
17	32	31	28	28	88	86	92	100
18	32	30	28	27	86	80	92	100
19	33	29	28	27	86	80	92	100
20	33	30	27	27	86	76	92	100
21	34	31	29	28	84	76	86	100
22	34	31	30	29	84	76	86	98
23	35	32	30	28	84	74	86	96

Table 6 (continued) Body weight and survival data

Pyridine

Week	Body weight (gms) (average individual)				Survival (%)			
	Male		Female		Male		Female	
	Low	High	Low	High	Low	High	Low	High
24	29	31	30	28	80	74	86	96
25	34	31	30	28	80	74	86	96
26	28	31	30	27	76	74	86	96
27	33	30	28	28	76	72	86	96
28	28	31	28	26	76	72	86	96
29	34	31	29	27	74	68	86	94
30	28	31	29	26	74	68	86	94
31	34	30	28	27	72	68	86	88
32	28	31	28	26	72	68	86	88
33	35	31	29	28	72	68	82	88
34	29	32	29	28	72	68	82	88
35	36	32	29	28	72	68	82	88
36	36	32	29	28	72	68	82	88
37	35	32	30	29	70	68	80	88
38	35	32	30	28	70	66	80	88
39	34	32	30	29	70	66	80	88
40	35	32	30	29	70	66	80	88
41	35	32	29	29	68	66	80	86
42	36	32	29	29	68	66	80	86
43	35	33	29	29	66	66	76	86
44	36	34	29	30	66	66	76	86
45	36	34	30	30	66	66	74	86

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Pyridine

Pyridine

Week	Body weight (gms) (average individual)				Survival			
	Male		Female		Male		Female	
	Low	High	Low	High	Low	High	Low	High
46	36	33	30	30	66	66	74	86
47	36	33	31	30	66	66	74	86
48	36	34	31	30	66	66	74	86
49	36	34	31	30	62	64	74	82
50	36	34	31	31	60	64	74	82
51	36	34	31	31	56	64	74	82
52	36	34	31	31	56	64	74	82
53	36	34	31	31	54	64	72	82
54	36	34	31	31	54	64	70	82
55	36	34	31	31	48	64	68	82
56	36	34	31	31	48	64	68	82
57	36	34	31	31	48	60	68	82
58	35	33	31	30	48	58	66	82
59	36	34	31	31	48	58	66	80
60	35	33	31	30	48	58	66	78

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Table 7. Body weight and Survival Data
Ethylene Glycol

Week	Body weight (gms) (average individual)				Survival (%)			
	Male		Female		Male		Female	
	Low	High	Low	High	Low	High	Low	High
1	16	17	14	15	100	100	100	100
2	22	21	18	19	100	100	100	100
3	24	26	22	20	100	100	100	100
5	27	27	23	22	100	100	100	100
6	28	28	24	23	100	100	100	100
7	29	29	25	24	100	100	100	100
8	30	30	25	24	100	100	98	100
9	30	30	26	25	100	100	98	100
10	30	31	26	25	100	100	98	100
11	30	31	26	25	100	100	98	100
12	30	31	26	26	100	100	98	100
13	32	32	26	25	98	100	98	98
14	32	32	26	25	96	100	98	96
15	32	31	26	25	96	100	98	96
16	32	31	26	26	94	98	96	94
17	32	30	26	26	--	94	96	94
18	32	30	27	26	94	92	92	94
19	32	30	26	25	94	90	92	94
20	32	31	27	26	94	86	92	94
21	33	32	27	--	94	86	92	94
22	35	31	--	27	92	84	92	92
23	34	33	28	28	92	84	90	92

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Table 7. Body weight and survival data

Week	Body weight (gms) (average individual)				Survival (%)			
	Male		Female		Male		Female	
	Low	High	Low	High	Low	High	Low	High
24	35	31	--	27	92	82	90	92
25	34	34	28	27	92	82	90	92
26	35	31	25	27	92	82	88	85
27	34	34	27	28	92	80	86	85
28	35	33	27	27	90	80	86	85
29	34	35	27	28	88	78	84	85
30	35	33	27	27	84	76	84	85
31	34	35	28	29	82	74	84	83
32	35	33	26	28	80	74	82	83
33	34	35	29	29	78	74	82	83
34	35	33	26	28	78	62	82	83
35	35	35	28	28	78	62	80	83
36	35	33	27	28	78	62	80	83
37	35	35	28	29	76	62	80	83
38	35	33	26	28	76	62	80	83
39	34	34	29	28	72	62	80	83
40	35	33	27	29	72	62	80	83
41	34	35	29	29	72	62	78	80
42	35	34	28	30	72	62	78	80
43	35	34	29	30	72	62	78	78
44	36	33	27	29	72	62	78	78
45	35	34	28	31	68	62	78	78

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Table 7 (continued) Body weight and survival data

Ethylene Glycol

Week	Body weight (gms) (average individual)				Survival (%)			
	Male		Female		Male		Female	
	Low	High	Low	High	Low	High	Low	High
46	36	34	28	30	68	62	78	78
47	35	34	30	32	68	62	78	78
48	35	36	29	30	68	62	76	76
49	35	36	30	31	68	62	76	76
50	35	36	30	30	66	62	76	76
51	35	36	30	30	66	60	76	76
52	36	36	31	30	66	60	76	76
53	36	36	31	30	66	60	76	76
54	35	36	30	30	66	60	76	72
55	35	36	30	30	66	60	76	72
56	35	36	30	30	66	60	76	68
57	35	36	30	30	64	60	76	68
58	35	36	30	30	64	58	74	68
59	36	36	30	30	62	56	74	66
60	36	36	30	30	60	54	72	66

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Table 8. Body weight and Survival Data

Week	Body weight (gms) (average individual)				Survival (%)			
	Male		Female		Male		Female	
	Low	High	Low	High	Low	High	Low	High
1	16	19	15	16	100	100	100	100
2	21	23	18	20	100	100	100	100
3	24	25	20	22	100	100	100	100
4	25	26	21	23	98	100	100	100
5	26	28	22	23	98	100	100	100
6	27	29	23	24	98	100	100	100
7	28	30	24	25	98	100	100	100
8	29	31	24	26	98	100	100	100
9	29	31	25	26	96	100	100	98
10	29	31	25	27	96	100	98	98
11	30	31	25	28	96	100	98	98
12	30	31	25	27	96	98	98	98
13	30	32	26	28	96	96	98	98
14	31	32	26	28	96	94	98	98
15	31	32	26	28	96	94	98	98
16	31	31	26	28	96	94	98	98
17	31	32	27	28	96	92	98	98
18	31	32	27	28	96	92	92	98
19	31	34	27	30	94	92	92	98
20	31	35	28	30	90	90	92	98
21	30	33	27	28	88	90	92	98
22	33	35	27	28	88	90	92	98
23	32	34	28	29	86	90	92	98

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Table 8. Body weight and Survival Data (cont.)

2-Chlorethanol

Week	Body weight - gms (average individual)				Survival (%)			
	Male		Female		Male		Female	
	Low	High	Low	High	Low	High	Low	High
24	32	36	29	28	79	90	92	98
25	31	34	27	29	79	88	92	96
26	31	36	28	29	79	84	92	96
27	31	34	28	29	79	82	88	96
28	31	35	28	29	77	78	88	96
29	31	33	32	29	77	78	88	96
30	33	34	29	30	74	76	88	96
31	32	32	27	29	74	74	86	96
32	33	34	30	30	74	74	86	96
33	32	33	28	30	72	70	86	96
34	34	35	30	31	74	66	86	96
35	32	32	28	30	74	64	86	96
36	34	35	31	31	74	64	86	94
37	33	33	31	30	74	62	86	94
38	34	33	31	31	74	60	86	92
39	33	35	31	31	72	60	86	90
40	34	33	31	32	70	60	86	90
41	32	34	31	31	68	60	86	90
42	34	33	31	32	66	60	86	90
43	33	33	33	31	66	60	86	90
44	33	33	32	32	66	60	86	90
45	33	34	33		66	60	86	

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2-Chlorethanol

Week	(average individual)				Survival (%)			
	Male		Female		Male		Female	
	Low	High	Low	High	Low	High	Low	High
46	33	33	32	32	62	60	86	90
47	33	34	33	32	62	60	86	90
48	34	34	33	33	60	60	84	88
49	33	34	32	32	58	60	84	88
50	35	35	34	34	58	60	84	88
51	33	35	30	34	55	58	84	88
52	34	34	31	33	55	58	84	88
53	34	34	30	33	53	58	84	88
54	35	35	32	33	53	58	84	88
55	35	35	32	34	51	58	84	88
56	35	35	32	33	51	58	80	88
57	35	35	32	33	51	58	80	88
58	35	35	33	32	51	58	80	88
59	36	35	32	33	49	58	78	88
60	35	35	32	32	49	56	78	88

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Table 9. Body weight and Survival Data
Benzethonium Chloride

Week	Body weight (ms) (average individual)				Survival (%)			
	Male		Female		Male		Female	
	Low	High	Low	High	Low	High	Low	High
1	17	18	17	15	100	100	100	100
2	21	21	20	18	100	100	100	100
3	24	24	22	20	100	100	100	100
4	26	27	23	21	100	100	100	100
5	27	28	24	22	100	100	100	100
6	29	29	25	23	100	100	100	100
7	30	29	26	24	98	100	98	98
8	30	29	27	25	98	100	98	98
9	30	29	26	26	98	98	98	98
10	30	28	26	26	98	96	98	98
11	31	28	27	26	98	94	94	98
12	32	30	27	26	98	92	98	98
13	32	32	28	26	98	78	98	94
14	32	31	28	26	98	78	98	94
15	32	32	28	25	98	76	98	94
16	32	31	28	25	98	74	98	92
17	31	30	27	26	98	74	96	88
18	31	28	27	25	98	72	96	88
19	33	30	28	26	94	68	94	86
20	35	30	30	26	92	68	94	84
21	32	33	28	28	92	66	94	84
22	34	33	29	28	88	64	94	82
23	32	33	28	28	88	64	94	82

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Body weight and Survival Data (cont.)

Benzethonium chloride

Week	Body weight - gms (average individual)				Survival (%)			
	Male		Female		Male		Female	
	Low	High	Low	High	Low	High	Low	High
24	33	29	28	28	88	62	84	82
25	32	33	26	27	86	62	84	80
26	33	29	28	27	86	62	84	80
27	31	33	25	28	84	62	84	78
28	33	29	28	26	84	62	82	78
29	32	32	28	26	80	62	78	74
30	33	30	29	27	80	60	76	72
31	33	32	28	28	78	60	76	72
32	34	29	30	28	74	58	76	72
33	33	33	29	27	72	52	76	72
34	35	30	30	29	72	52	76	72
35	33	34	29	28	76	50	76	72
36	33	34	31	28	70	50	76	72
37	33	34	30	29	70	50	76	68
38	34	34	31	28	70	50	76	68
39	34	34	31	29	70	50	76	64
40	34	34	31	29	70	50	76	64
41	34	34	30	29	70	50	76	64
42	34	34	32	30	66	50	74	64
43	34	34	31	30	66	48	74	64
44	34	34	33	30	66	48	74	64
45	34	34	32	30	66	48	74	60

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Table 9 (continued) Body weight and survival Data

Benzethonium chloride

Week	Body weight (gms) (average individual)				Survival (%)			
	Male		Female		Male		Female	
	Low	High	Low	High	Low	High	Low	High
46	34	34	33	29	64	46	74	60
47	34	35	32	30	64	46	74	60
48	34	35	33	29	62	46	74	60
49	34	35	32	30	62	42	74	60
50	35	35	33	30	62	42	74	60
51	34	36	32	30	58	42	74	60
52	34	35	32	29	56	42	74	54
53	34	36	32	30	54	42	74	54
54	35	35	33	30	54	42	74	54
55	34	35	32	30	54	42	74	54
56	34	34	32	30	52	42	74	54
57	34	35	32	30	52	42	74	54
58	34	35	32	30	52	40	74	54
59	34	35	32	30	48	40	74	52
60	34	34	32	30	44	40	72	50

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Experimental and Survival Data

Experiment

Week	Body weight (gms) (average individual)				Survival (%)			
	Male		Female		Male		Female	
	Low	High	Low	High	Low	High	Low	High
1	17	19	16	17	98	100	100	100
2	22	23	19	20	98	100	100	100
3	24	24	21	21	98	100	100	100
4	25	26	22	22	98	100	100	100
5	26	27	23	23	98	100	100	100
6	27	28	24	25	98	100	100	100
7	28	29	25	26	98	100	98	100
8	28	29	25	26	98	100	96	100
9	28	30	26	26	98	100	96	100
10	29	30	26	26	98	100	96	100
11	29	31	25	27	98	98	94	100
12	29	31	25	27	98	98	94	98
13	31	31	26	28	96	98	92	98
14	31	31	26	28	96	96	92	98
15	32	32	28	28	96	96	90	98
16	33	32	28	28	94	90	90	98
17	32	32	28	27	92	90	90	98
18	31	32	26	26	92	90	88	98
19	32	32	26	27	92	90	88	98
20	32	32	25	27	92	90	88	98
21	31	33	26	--	88	86	86	96
22	33	33	25	27	88	86	83	94
23	33	33	25	28	86	82	83	94

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Table 10. Body weight and survival data (continued)
Phenol Red

Week	Body weight (gms) (average individual)				Survival (%)			
	Male		Female		Male		Female	
	Low	High	Low	High	Low	High	Low	High
24	32	33	27	27	86	76	83	94
25	33	33	25	29	84	76	83	94
26	34	33	28	28	82	76	83	94
27	33	32	26	30	82	72	83	94
28	34	33	29	28	82	66	83	94
29	31	32	27	30	78	64	83	94
30	32	33	29	29	76	64	83	94
31	31	33	27	31	74	60	83	94
32	33	33	29	28	72	60	83	92
33	31	33	28	31	72	60	81	92
34	34	34	30	29	70	60	81	92
35	33	34	28	31	70	60	81	92
36	35	34	29	30	68	60	81	92
37	34	34	27	31	68	60	81	92
38	35	34	29	32	68	60	81	92
39	33	33	28	31	68	60	81	92
40	34	34	30	30	66	70	81	92
41	33	34	27	32	66	60	81	92
42	34	34	30	31	64	60	81	88
43	33	34	29	33	64	60	81	88
44	35	35	31	32	60	60	79	88
45	34	35	30	33	60	60	79	88

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Phenol Red

Week	(average individual)				Survival (%)			
	Male		Female		Male		Female	
	Low	High	Low	High	Low	High	Low	High
46	34	36	31	32	60	60	79	86
47	34	35	29	34	60	60	79	84
48	36	35	31	33	58	60	77	82
49	35	35	30	34	52	60	77	82
50	36	36	31	33	50	60	77	82
51	35	36	32	33	50	58	77	82
52	36	37	33	33	50	58	77	82
53	35	35	33	33	50	58	77	82
54	35	34	34	33	50	58	77	82
55	35	35	33	33	46	56	77	82
56	36	35	33	33	42	56	77	78
57	35	35	33	33	40	54	77	78
58	35	34	33	33	38	54	77	78
59	35	34	33	33	38	52	77	78
60	35	33	33	33	36	52	75	76

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Table 11. Body Weight and Survival Data

Thimerosal, N.F.

Week	Body weight (gms) (average individual)				Survival (%)			
	Male		Female		Male		Female	
	Low	High	Low	High	Low	High	Low	High
1	16	16	15	16	100	100	100	100
2	21	21	19	18	100	100	100	100
3	23	24	21	20	100	100	100	100
4	26	25	23	21	100	100	100	100
5	27	27	23	22	100	100	100	100
6	28	27	24	23	100	100	100	100
7	29	27	25	24	100	100	98	98
8	30	28	26	25	100	100	98	98
9	33	27	27	25	100	100	98	98
10	31	27	27	25	100	100	98	98
11	31	26	27	25	100	100	96	98
12	31	26	27	25	100	96	96	98
13	31	26	26	26	100	88	96	96
14	32	27	26	26	100	86	96	96
15	32	27	28	25	100	84	92	96
16	32	27	28	25	98	84	90	96
17	32	27	28	26	98	84	90	94
18	32	28	28	26	96	82	90	94
19	32	29	29	26	96	82	90	94
20	32	28	29	26	96	82	88	94
21	33	29	29	26	96	82	84	92
22	34	29	29	27	96	82	84	88
23	34	29	28	25	96	82	84	88

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 Table 11. Body weight and survival data (continued)
 Thimerosal, N.F.

Week	Body weight (gms) (average individual)				Survival (%)			
	Male		Female		Male		Female	
	Low	High	Low	High	Low	High	Low	High
24	34	29	31	29	96	82	84	88
25	33	29	28	25	92	82	84	88
26	32	30	30	29	92	82	84	88
27	33	29	29	26	92	82	84	88
28	32	30	31	29	92	82	84	88
29	33	30	29	26	92	82	84	88
30	33	29	30	29	90	82	84	88
31	33	30	31	26	90	82	84	88
32	33	31	31	28	90	82	84	88
33	33	30	30	27	90	82	84	88
34	32	30	31	27	90	82	84	86
35	34	30	29	26	90	82	84	84
36	32	30	31	28	90	82	84	84
37	34	30	30	28	88	82	84	84
38	34	30	30	29	84	82	84	82
39	34	30	31	29	84	82	82	82
40	33	30	31	30	84	82	80	82
41	34	31	33	30	82	78	80	82
42	34	31	31	30	74	76	80	82
43	35	31	32	30	74	74	80	80
44	33	30	31	30	72	74	74	80
45	35	31	33	30	72	74	74	80

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Table 11 (continued) Body weight and survival data

Thimerosal, N.F.

Week	(average individual)				Survival (%)			
	Male		Female		Male		Female	
	Low	High	Low	High	Low	High	Low	High
46	35	31	32	29	70	74	74	80
47	36	31	32	29	70	74	74	80
48	35	31	32	30	70	66	72	80
49	35	31	32	30	68	64	72	80
50	35	31	32	30	68	64	70	80
51	35	30	32	30	68	64	70	80
52	35	31	33	29	68	62	70	80
53	35	31	33	30	68	60	70	80
54	35	31	33	29	66	60	68	80
55	35	31	33	29	66	60	68	80
56	35	31	33	29	66	60	68	78
57	35	31	33	30	66	60	66	78
58	35	31	33	29	64	60	66	78
59	35	31	33	30	62	60	66	78
60	35	31	33	29	60	60	66	78

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Table 12. Body Weight and Survival Data
Methyl Parasept

Week	(average individual)				Survival (%)			
	Male		Female		Male		Female	
	Low	High	Low	High	Low	High	Low	High
1	18	18	16	16	100	100	100	100
2	22	22	20	20	100	100	100	100
3	24	24	22	22	100	100	100	100
4	26	26	22	22	100	100	100	100
5	27	27	24	23	100	100	100	100
6	28	28	24	25	100	100	100	100
7	29	29	25	25	98	100	100	100
8	30	30	26	26	98	100	100	100
9	30	31	27	26	98	100	100	100
10	30	31	27	27	98	100	100	100
11	31	31	28	27	96	100	100	100
12	31	32	28	27	96	100	100	100
13	32	31	28	27	96	100	100	100
14	32	32	28	28	96	100	100	100
15	33	32	29	28	96	100	100	100
16	33	32	28	27	96	100	100	100
17	32	33	29	27	96	100	100	100
18	33	33	28	27	96	100	100	100
19	32	33	28	27	96	94	100	98
20	33	33	29	27	96	94	100	98
21	33	33	28	28	94	92	100	98
22	33	33	29	29	94	92	96	98
23	33	33	28	30	92	92	96	

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Table 12. Body weight and survival data

Methyl Parasept

Week	Body weight (gms) (average individual)				Survival (%)			
	Male		Female		Male		High	
	Low	High	Low	High	Low	High	Low	High
24	33	32	29	26	92	92	96	98
25	34	33	28	29	92	92	96	98
26	33	33	28	25	90	90	96	96
27	34	34	28	28	88	88	96	96
28	34	33	28	25	86	88	96	96
29	34	33	29	28	84	88	93	94
30	34	34	29	26	84	88	93	92
31	34	33	28	29	81	88	93	82
32	34	34	29	26	80	88	93	82
33	33	32	29	28	80	86	91	80
34	34	35	29	27	80	86	91	72
35	35	33	29	31	80	86	89	72
36	35	34	30	28	80	82	89	72
37	34	34	30	28	80	80	89	72
38	35	33	30	29	78	80	89	72
39	34	34	30	29	78	80	89	70
40	36	33	30	29	78	80	89	70
41	34	34	31	29	78	80	89	70
42	36	34	31	29	74	80	89	70
43	35	35	32	29	74	80	89	70
44	36	35	32	29	72	78	89	70
45	35	35	31	29	70	78	87	68

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Methyl parasept

Week	Body weight (gms) (average individual)				Survival (%)			
	Male		Female		Male		Female	
	Low	High	Low	High	Low	High	Low	High
46	36	35	31	29	70	78	87	66
47	35	35	32	29	68	78	87	68
48	36	35	32	29	62	78	87	68
49	35	34	32	30	60	76	87	66
50	36	35	32	30	60	74	87	66
51	35	35	32	31	58	72	87	66
52	35	35	32	32	56	70	87	66
53	35	35	32	32	56	70	87	66
54	35	35	32	33	56	68	87	66
55	35	35	32	32	56	68	87	66
56	35	35	32	32	56	66	87	66
57	35	35	32	31	56	66	85	66
58	35	35	32	32	54	66	85	66
59	35	35	32	31	54	66	83	64
60	35	35	32	30	48	64	83	62

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Table 13. Body Weight and Survival Data

1, 2, 5, 6 Dibenanthracene in corn oil

Week	Body weight (gms) (average individual)				Survival (%)			
	Male		Female		Male		Female	
	Low	High	Low	High	Low	High	Low	High
1	17	18	15	16	100	100	100	100
2	21	22	19	19	100	100	100	100
3	24	24	21	21	100	100	100	100
4	26	26	22	22	100	100	100	100
5	27	28	23	23	100	100	100	100
6	28	29	24	24	100	100	100	100
7	29	29	24	24	100	100	98	100
8	29	30	25	25	100	100	98	100
9	29	30	26	26	100	100	98	100
10	30	30	26	26	100	100	98	100
11	31	31	26	26	100	100	98	100
12	31	32	26	27	98	100	98	100
13	32	33	27	28	98	100	96	98
14	32	33	27	28	98	100	94	98
15	33	34	28	28	98	100	94	96
16	33	34	28	28	98	96	94	96
17	33	34	27	29	98	96	94	88
18	33	34	28	28	96	96	92	86
19	33	35	27	28	96	96	86	86
20	32	35	27	27	94	92	78	84
21	34	35	27	28	90	90	76	82
22	34	35	28	27	86	84	70	80
23	34	34	28	28	84	84	70	68

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 Table 13. Body weight and survival data (continued)
 100 mg/kg of 3-methylcholanthrene in corn oil

Week	Body weight (gms) (average individual)				Survival (%)			
	Male		Female		Male		Female	
	Low	High	Low	High	Low	High	Low	High
24	34	34	28	27	80	80	70	68
25	34	34	28	27	78	78	70	68
26	34	36	27	26	74	70	70	66
27	34	35	28	28	74	70	70	58
28	32	36	28	25	74	68	70	58
29	34	34	29	27	70	68	70	58
30	27	35	28	26	70	66	70	58
31	34	35	29	27	68	56	70	56
32	28	36	28	27	66	56	70	56
33	34	35	29	29	64	54	70	52
34	32	36	30	26	62	54	70	52
35	35	36	30	28	62	50	70	52
36	35	35	30	27	62	48	68	52
37	33	36	31	28	62	44	68	52
38	35	36	31	28	62	44	68	52
39	34	37	31	28	56	42	68	52
40	34	37	31	28	56	40	68	52
41	34	37	31	29	54	40	68	52
42	36	37	32	30	54	38	68	52
43	35	36	31	30	54	36	64	52
44	36	36	31	30	54	36	64	52
45	35	37	32	30	54	36	64	52

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Table 13 (continued) Body weight and survival data

1, 2, 5, 6 Dibenanthracene in corn oil

Week	Body weight (gms) (average individual)				Survival (%)			
	Male		Female		Male		Female	
	Low	High	Low	High	Low	High	Low	High
46	36	36	33	29	54	36	64	52
47	35	36	32	31	52	36	62	52
48	36	37	32	30	52	36	62	52
49	36	36	33	30	52	36	60	52
50	35	36	32	29	52	36	60	52
51	36	36	31	31	52	34	60	52
52	35	36	31	31	50	34	60	52
53	36	36	32	31	48	34	60	52
54	36	36	33	31	46	34	58	50
55	36	36	33	31	46	34	58	50
56	36	36	32	31	46	34	58	48
57	36	36	33	31	46	34	58	48
58	36	36	32	31	46	32	58	48
59	36	36	32	31	46	32	58	44
60	36	36	32	31	46	32	58	44

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Table 14. Body Weight and Survival Data

Week	Body weight (gms) (average individual)				Survival (%)			
	Male		Female		Male		Female	
	Low	High	Low	High	Low	High	Low	High
1	11	16	15	14	100	100	100	100
2	19	20	18	18	100	100	100	100
3	22	23	20	19	100	100	100	100
4	24	25	21	20	100	100	100	100
5	26	27	22	22	100	100	100	98
6	27	28	24	23	100	100	100	98
7	27	28	24	23	100	100	100	98
8	27	28	25	24	100	98	100	98
9	27	28	25	25	96	96	100	96
10	29	28	25	25	92	92	100	96
11	31	29	26	25	90	92	100	96
12	32	30	27	26	88	92	100	96
13	31	30	26	26	88	88	100	96
14	33	30	28	27	88	88	100	96
15	30	31	26	27	86	86	100	96
16	30	31	26	27	86	86	100	96
17	31	31	26	26	86	86	100	96
18	31	31	27	29	86	86	100	96
19	33	32	27	29	86	86	100	96
20	30	31	27	28	84	82	96	96
21	34	31	28	29	82	78	93	96
22	31	32	24	27	82	76	93	96
23	34	32	28	29	82	76	93	96

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Table 14. Body weight and survival data

1, 2, 5, 6 Dibenanthracene in saline

Week	Body weight (gms) (average individual)				Survival (%)			
	Male		Female		Male		High	
	Low	High	Low	High	Low	High	Low	High
24	31	32	24	26	80	74	93	94
25	35	32	29	29	80	74	91	94
26	32	33	25	27	78	72	91	92
27	35	31	31	28	78	72	91	92
28	32	33	26	28	76	72	89	92
29	34	31	30	30	74	72	89	92
30	32	33	26	28	74	72	89	92
31	35	32	29	30	72	72	87	92
32	32	33	25	27	68	70	87	92
33	35	33	30	30	68	70	84	92
34	33	34	27	28	68	68	80	92
35	35	32	31	30	68	68	80	92
36	33	33	27	29	66	68	80	92
37	34	32	29	31	66	66	80	92
38	34	33	30	30	62	64	80	92
39	35	34	30	31	62	64	78	90
40	34	34	29	30	62	64	78	90
41	35	33	30	32	62	60	78	90
42	35	33	30	31	60	60	76	90
43	35	33	30	30	60	60	76	90
44	34	34	29	30	58	60	76	90
45	36	33	30	31	54	60	76	90

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1, 2, 5, 6 dibenzanthracene in saline

Week	Body weight (gms) (average individual)				Survival (%)			
	Male		Female		Male		Female	
	Low	High	Low	High	Low	High	Low	High
46	35	33	30	30	54	60	72	90
47	36	34	31	31	54	60	72	88
48	35	35	30	31	52	60	72	86
49	36	33	30	31	52	60	70	86
50	35	35	30	32	50	56	70	86
51	35	34	30	32	50	54	70	86
52	35	35	30	32	48	54	70	86
53	35	34	30	32	46	54	70	86
54	35	35	30	32	46	54	70	86
55	34	35	30	31	44	52	70	86
56	34	35	30	32	44	52	68	86
57	34	35	30	31	42	52	68	86
58	34	35	30	31	40	52	68	86
59	34	35	30	31	40	52	68	86
60	34	35	30	31	40	52	68	86

Table Explanation

Tables 15 through 51
Titled "Fate of Mice and Incidence of Neoplasm"

Table No.

Fate of Mice⁽¹⁾ and Incidence of Neoplasm

Group Identification

<u>Animal Number</u> (2)	<u>Fate</u> (3)	<u>Weeks on test</u> (4)	<u>Tissue collected</u> (5)	<u>Neoplasm observed</u> (6)	
				Gross (location)	Histologic (type)

- (1) This set of tables covers only animals which died on test.
- (2) Animal Number denotes the number assigned to each animal at the time they were weaned. Each weanling was identified by ear notches and assigned an identification number. An * asterick by the animal number indicates the animal was one selected at random for histologic examination of tissue at termination. Tissues scheduled for examination included lung, heart, liver, spleen, gonad, uterus (female), kidney and site of injection.
- (3) Fate denotes what happened to the animal according to the following code:
d - died, found dead in cage and at least examined grossly
c - cannibalized; found missing from cage and no gross examination
k - killed because of moribund condition, large tumor or other condition that indicated mouse could die in the next few days.
- (4) Weeks on test is also the age of the animal at time of death since treatment was applied within 24 hours of birth.
- (5) Tissues collected denotes the handling of tissues in terms of collection and fixing for histologic examination. In the original contract and through the first portion of the experiment only grossly observed suspected neoplasms were collected and fixed for histologic examination. With the increased mortality in the first half of the study and in an effort to obtain more material for histologic examination, all animals which did not show advanced autolysis were placed (whole body) in fixative. The following code identifies the tissues collected:
N - None; S - Single tissue, abnormal or suspected tumor; T - Total animal
- (6) Neoplasms observed
Gross (location) Any enlargement or abnormality which could be suspected of being neoplastic was noted and location given. Notation was made only where suspected neoplasm was observed.
Histologic (type) Observations are noted with regard to neoplasms only and a notation made in all cases where histologic examination made whether a tumor was present or not.

Type of tumor is noted and also location if information not noted in "Gross (location)" column. If suspected gross tumor was shown by histology to not be neoplastic, this is noted and explained.

Table 15

Fate of Mice and Incidence of Neoplasia

Negative Control - Males

Animal Number	Fate ⁽¹⁾	Weeks on test	Tissues ⁽²⁾ collected	Neoplasms Observed	
				Gross (location)	Histologic (type)
5032	d	8	N		
5016	d	9	N		
5033	d	10	S		
4896	d	12	N		
4926	d	12	S		
4927	d	12	N		
5017	d	13	N		
4925	d	13	S		
4955	d	14	N		
4916	d	14	S		
4857	d	14	S		
4890	d	14	S		
5015	d	14	N		
4905	d	15	N		
4972	d	15	S		
4974	d	15	N		
4884	d	15	S		
5027	d	15	N		
5010	d	16	N		
5013	d	16	N		
4971	d	16	N		
4973	d	17	N		
4938	d	17	N		
4848	d	18	N		
4871	d	18	N		
4901	d	18	N		
4900	d	18	N		
4950	d	20	N		
4880	d	20	N		
4881	d	20	N		
4847	d	20	N		
5041	d	21	N		
4984	d	21	N		
4893	d	22	N		
5000	d	23	N		
4865	d	25	N		
5001	d	25	N		
4949	d	28	N		
4875	d	29	N		
4878	d	29	N		
4911	d	29	N		
4920	d	29	N		

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Table 15 (continued)

Fate of Mice and Incidence of Neoplasm

Animal Number	Fate (1)	Weeks on test	Tissues collected (2)	Neoplasms observed	
				Gross (location)	Histologic (type)
4851	d	31	N		
4852*	d	31	N		Negative
4850	d	33	N		
4915	d	33	N		
4892	d	34	N		
4846	d	35	N		
4954	k	35	N		
4864	k	35	N		
4889	d	35	N		
5007	c	36	N		
5019	d	36	N		
4869	k	37	N		
4872	k	37	N		
4876	d	37	N		
4914	k	37	T		
4921*	k	37	T		Negative
4867	d	37	N		
4862	d	39	N		
4845	d	39	N		
4924	d	43	N		
5037	c	43	N		
5044	k	43	N		
5042	c	45	N		
4913	d	45	T		
4853	d	45	N		
4854*	k	45	T		Negative
4988	k	46	N		
4877*	d	47	N		Negative
4863*	k	47	T		Negative
4873*	k	47	T		Spleen & kidney
5043*	k	47	T		Lymphoma
5005 *	k	48	T		Spleen lymphoma
5040	d	48	N		Negative
4970	d	48	T		
4859	d	49	N		
5008	d	49	N		
4976	d	50	N		
5002*	d	51	T		Negative
4951	d	51	T		
4860	d	52	T		
5028	d	52	N		
4899	d	50	N		Not neoplastic
5031*	k	53	T	Subcutaneous	Subcutaneous ab- scess

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Fate of Mice and Incidence of Neoplasm

Negative control - Mice

Animal Number	Fate ⁽¹⁾	Weeks on test	Tissues ⁽²⁾ collected	Neoplasms observed	
				Gross (location)	Histologic (type)
4888	d	61	N		
4956	d	53	N		
4997*	k	54	T		Negative
4948	d	56	T		
4963	d	56	N		
4894*	d	57	T		Negative
4983*	d	56	T		Negative
4957	d	53	T		
4887	d	62	N		
5006	d	58	T	Lung	Extensive pneumonia
4977	d	58	T		
4879	d	59	T		
5028	d	55			
4862*	d	61	T		Negative
4868*	k	61	T	Subcutaneous	Not neoplastic, granuloma
4874*	d	61	T		Lymphoma of spleen, hepatitis
5009*	d	60	T		Negative
4932*	d	62	T	Lung	Not neoplastic, chronic pneumonia

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Table 16

Fate of Mice and Incidence of Neoplasm

Negative control - Females

Animal Number	Fate ⁽¹⁾	Weeks on test	Tissues ⁽²⁾ collected	Neoplasms observed	
				Gross(location)	Histologic (type)
5195	d	5	N		
5196	d	5	N		
5129	d	11	S		
5211	d	11	N		
5140	d	12	S		
5118	d	14	S		
5144	d	14	N		
5049	d	15	S		
5058	d	15	N		
5120	d	15	N		
5232	d	16	N		
5161	d	16	N		
5143	d	16	N		
5117	d	18	N		
5073	d	19	N		
5074	d	19	S		
5220	d	20	N		
5047	d	20	N		
5201	d	22	N		
5207	d	22	N		
5216	c	22	N		
5218	c	22	N		
5190	d	22	N		
5219	d	23	N		
5069	d	23	N		
5187	d	24	N		
5200	d	26	N		
5131*	c	32	N		Negative
5099	d	33	N		
5046	d	35	S		
5051	d	35	T		
5052	d	35	N		
5204	d	36	N		
5221	c	36	N		
5212*	d	38	T		Negative
5058	d	39	N		
5213*	d	42	T		Negative
5209*	d	44	T		Negative
5086	d	46	N		
5065	d	47	N		

Table 16 (continued)

Negative control - females

Animal Number	Fate (1)	Weeks on test	Tissues (2) collected	Neoplasms observed	
				Gross (location)	Histologic (type)
5194	d	48	N		
5231	d	49	N		
5224	d	49	N		
5084	d	50	N		
5188 *	k	52	T		Lymphomatous kidney, not neoplastic
5147*	k	53	T	Subcutaneous	Subcutaneous abscess
5106*	d	51	T	Kidney	Cystic nephritis
5093*	d	52	T		Mixed adenoma & adeno- of lung; lymphoma of spleen
5141	d	54	S		
5214*	k	56	T		Negative
5139	d	56	T		
5198*	d	58	T	Lung	Extensive pneumonia, not neoplastic
5068*	d	63	T		Negative
5199*	d	56	T	Lung	Not neoplastic, abscess
5072	d	65	T	Lung	
5067*	d	65	T		Negative

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Table 17

Fate of Mice and Incidence of Neoplasm

Pyridine - low level - males

Animal Number	Fate ⁽¹⁾	Weeks on test	Tissues ⁽²⁾ collected	Neoplasms observed	
				Gross (location)	Histologic (type)
5975	d	7	N		
5971	d	10	S		
5946	d	12	S		
5944	d	13	N		
5964	d	13	S		
5945	d	17	S		
5974	d	18	N		
5957	d	23	N		
5931	d	24	N		
5933	d	24	N		
5930	d	25	N		
5929	d	25	N		
5977*	k	29	T		Negative
5936	d	31	T		
5968*	d	37	T		Negative
5943*	d	41	T		Negative
5939*	d	43	T		Spleen & subc. lympho- sarcoma
5966	d	49	N		
5963	d	49	N		
5962*	d	50	T		Negative
5934*	k	55	T		Negative
5937*	k	55	T	Lung	Lung abscess
5976*	d	49	T	Kidneys neoplastic	Nephritis & granulation
5970*	d	50	T	Lung	Lung abscess
5961	k	51	T		
5960*	k	55	T		Negative

Table 18

Fate of Mice and Incidence of Neoplasm

Pyridine - low level - females

Animal Number	Fate ⁽¹⁾	Weeks on test	Tissues ⁽²⁾ collected	Neoplasm observed	
				Gross (location)	Histologic (type)
5997	d	15	N		
6005	d	15	N		
5980	d	17	N		
5995	d	17	N		
6006	d	18	N		
5989	d	21	N		
5993	d	21	N		
6004	k	33	N		
6007*	k	33	T		Negative
5992*	d	37	T		Not neoplastic; lung abscess
6015	d	43	N		
6016	d	43	N		
6017	d	45	N		
6003	d	49	N		
6021*	d	53	T		Negative
5983*	d	53	T		Negative
5994	d	58	T		
5979*	d	63	T	Lung	Not neoplastic; lung abscess
6027	d	61	T		

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Table 19

Fate of Mice and Incidence of Neoplasm

Pyridine - high level - males

Animal Number	Fate (1)	Weeks on test	Tissues (2) collected	Neoplasm observed	
				Gross (location)	Histologic (type)
5870	d	11	S		
5869	d	12	S		
5868	d	12	S		
5845	d	13	S		
5843	d	15	N		
5831	d	17	N		
5836	d	17	N		
5840	d	17	N		
5855	d	18	N		
5856	d	18	N		
5860	d	18	N		
5838	d	20	N		
5867	d	20	N		
5835	d	23	N		
5851	d	29	N		
5853	d	29	N		
77	d	38	N		
5852	d	49	N		
5861*	k	59	T	Spleen, lymphomatous Urinary bladder, fibroma	
5849*	k	59	T		
5874	d	58	N		

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Table 20

Fate of Mice and Incidence of Neoplasm

Pyridine - high level - females

Animal Number	Fate ⁽¹⁾	Weeks on test	Tissues ⁽²⁾ collected	Neoplasm observed	
				Gross (location)	Histologic (type)
5891	d	23	N		
5904	d	23	N		
5887	d	29	N		
5886	d	30	N		
5913	d	31	N		
5884	d	33	N		
5922*	d	41	T		Lymphoma of liver, spleen kidney, uterus, LN
5918	d	49	N		
5924	d	49	N		
5905	d	59	N		
5893*	d	63	T		Negative
5882*	d	63	T		Negative

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Table 21

Fate of Mice and Incidence of Neoplasm

Ethylene glycol - low level - males

Animal Number	Fate (1)	Weeks on test	Tissues (2) collected	Neoplasm observed	
				Gross (location)	Histologic (type)
6691	d	13	N		
6693	d	13	S		
6695	d	14	S		
6692	d	22	N		
6715	d	28	N		
6711	d	29	N		
6712	d	30	N		
6701	d	31	N		
6673	d	31	N		
6714	d	32	N		
6702	d	33	N		
6682	d	37	N		
6686	d	39	N		
6688	d	39	N		
6704	d	45	N		
6671	d	45	N		
6676	d	49	N		
6703*	k	57	T		Negative
6675*	d	63	T		Negative

Table 22

Fate of Mice and Incidence of Neoplasm

Ethylene glycol - low level - females

Animal Number	Fate ⁽¹⁾	Weeks on test	Tissues ⁽²⁾ collected	Neoplasms observed	
				Gross (location)	Histologic (type)
6741	d	8	N		
6725	d	16	S		
6723	d	18	S		
6749	d	18	N		
6724	d	19	N		
6767	d	23	N		
6760	d	25	N		
6769	d	26	N		
6758	c	29	N		
6766*	d	32	T		Lung abscess & granuloma
6730	d	35	N		
6731	d	41	N		
6728*	d	49	T		Hemangioma ovary & lung
6770*	d	58	T	Lung	Abscess in pleural cavity
6754*	d	59	T		Negative

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Table 23

Fate of Mice and Incidence of Neoplasm

Ethylene glycol - high level - males

Animal Number	Fate ⁽¹⁾	Weeks on test	Tissues ⁽²⁾ collected	Neoplasms observed	
				Gross (Location)	Histologic (type)
6029	d	16	N		
6030	d	17	N		
6046	d	17	N		
6061	d	18	N		
6033	d	19	N		
6036	d	20	N		
6068	d	20	N		
6060	d	22	N		
6032	d	24	N		
6065	d	27	N		
6045	k	28	N		
6067	d	31	N		
6051	d	34	N		
6074	d	34	N		
6075	d	34	N		
6076	d	34	N		
6077	d	34	N		
6078	d	34	N		
6053*	d	50	T		Negative
6035*	d	61	T		Negative
6043*	d	61	T		Negative
6048*	d	60	T		Negative
6063	d	61	T		

Table 24

Fate of Mice and Incidence of Neoplasm

Ethylene glycol -high level - females

Animal Number	Fate ⁽¹⁾	Weeks on test	Tissues ⁽²⁾ collected	Neoplasms observed	
				Gross (location)	Histologic (type)
6105	d	13	S		
6080	d	14	S		
6107	d	16	N		
6084	d	18	N		
6106	d	22	N		
6079	d	23	N		
6095	d	25	N		
6085	d	31	N		
6125	d	41	N		
6090	d	43	N		
6102	d	50	T		
6110*	d	51	T		Negative
6109*	d	54	T		Lymphomatous infiltration of lung with necrosis
6113	d	54	T		
6094*	k	57	T	Lung	Not neoplastic; lung abscess
4108*	k	57	T	Subcu	Squamous cell carcinoma
098*	d	60	T		Negative

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Table 25:

Fate of Mice and Incidence of Neoplasm

2-Chlorethanol - low level - males

Animal Number	Fate ⁽¹⁾	Weeks on test	Tissues ⁽²⁾ collected	Neoplasms observed	
				Gross (location)	Histologic (type)
6906	d	4			
6893	d	19	N		
6889	d	20	N		
6880	d	20	N		
6894	d	21	N		
6909	d	23	N		
6895	d	24	N		
6892	d	24	N		
6885	d	24	N		
6915	d	28	N		
6907	d	30	N		
6898*	k	33	T		Lymphosarcoma, spleen
6899*	d	39	T		Negative
6917*	k	40	T		Negative
6878	d	41	N		
6908	k	42	N		
6904*	k	46	T		Negative
6877	d	49	N		
6882*	k	51	T		Lymphosarcoma
4*	d	48	T	Lung	Extensive pneumonia
6902*	d	52	T	Lung	Lung abscess
6913*	d	57	T		Negative
6888*	d	59	T		Negative

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Table 26

Fate of Mice and Incidence of Neoplasm

2-Chlorethanol - low level - females

Animal Number	Fate ⁽¹⁾	Weeks on test	Tissues ⁽²⁾ collected	Neoplasms observed	
				Gross (location)	Histologic (type)
6922	d	17	N		
6930	d	18	N		
6931	d	18	N		
6948	d	18	S		
6943	d	27	N		
6945	c	27	N		
6951	d	48	N		
6928*	k	55	T		Hemangioma of ovary
6921*	d	55	T		Lymphosarcoma, liver & spleen

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Table 27

Fate of Mice and Incidence of Neoplasm

2-Chlorethanol - high level - males

Animal Number	Fate (1)	Weeks on test	Tissues ⁽²⁾ collected	Neoplasms observed	
				Gross (location)	Histologic (type)
6784	d	13	N		
6792	d	13	S		
6819	d	14	S		
6785	d	17	N		
6776	c	20	N		
6780	d	25	S	Skin	Hyperkeratosis, sheath of
6813	d	26	N		
6811	d	26	N		
6809	c	27	N		
6812	d	28	N		
6779	d	28	N		
6814	k	30	N		
6778	d	31	N		
6786	d	33	N		
6787*	d	33	T		Negative
6800	d	34	N		
6796	d	34	N		
6788	c	35	N		
6789	d	35	N		
6793	d	38	N		
6797*	d	50	T	Lung neoplasm	Lung adenoma
6772*	d	63	T		Negative

Table 28

Fate of Mice and Incidence of Neoplasm

2-Chlorethanol - high level - females

<u>Animal Number</u>	<u>Fate (1)</u>	<u>Weeks on test</u>	<u>Tissues (2) collected</u>	<u>Neoplasms observed</u>	
				<u>Gross (location)</u>	<u>Histologic (type)</u>
6847	d	9	N		Negative
6860*	d	26	T		
6830*	d	36	T	Hemangioma of intestine	Not neoplastic
6857*	d	36	T		Negative
6852*	d	38	T		Lymph sarcoma of spleen kidney, liver and L.N.

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Table 29

Fate of Mice and Incidence of Neoplasia

Benzethonium - low level - males

Animal Number	Fate (1)	Weeks on test	Tissues (2) collected	Neoplasms observed	
				Gross (location)	Histologic (type)
7098	k	6	N		
7077	d	19	N		
7107	d	19	N		
7101	d	20	N		
7099	d	22	N		
7100	d	22	S		
7064	d	24	S		
7097	d	27	N		
7095	c	29	N		
7096	d	29	N		
7093*	k	29	T		Negative
7086	d	32	N		
7068	d	32	N		
7111	d	36	N		
7109	d	42	N		
7110*	k	42	T		Negative
7112*	k	44	T		Negative
7063	d	48	N		
7088	d	50	N		
7078*	d	52	N		Negative
7090*	d	53	T		Negative
7085*	k	52	T	Kidney	Suppurative nephritis
7061*	d	54	T		Negative
7072*	d	60	T		Negative
7071*	d	60	T		Negative

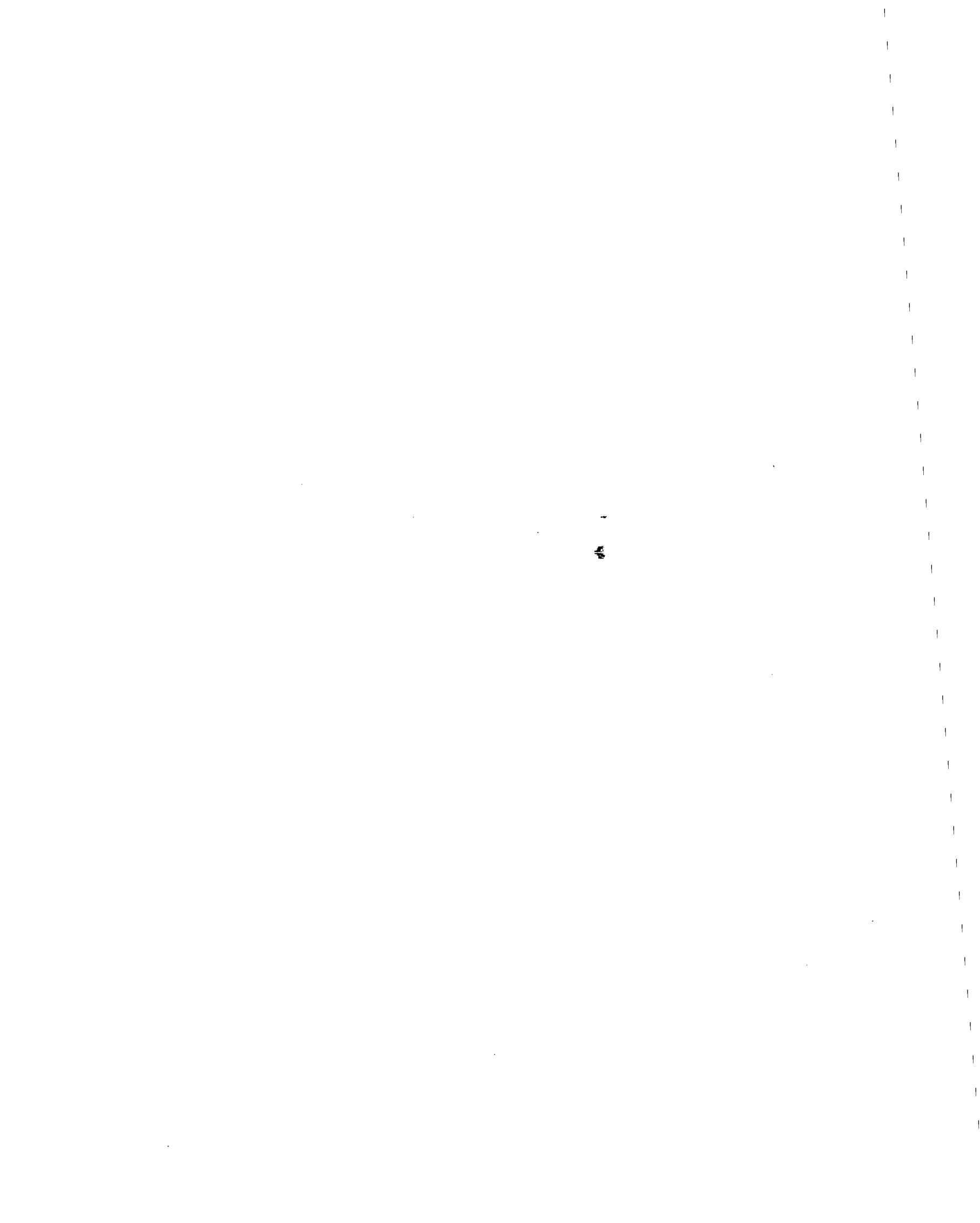


Table 30

Fate of Mice and Incidence of Neoplasm

Benzethonium - low level - females

Animal Number	Fate (1)	Weeks on test	Tissues ⁽²⁾ collected	Neoplasms observed	
				Gross (location)	Histologic (type)
7162	k	7	N		
7150	d	17	S		
7160	d	19	N		
7115	d	24	N		
7128	d	24	N		
7134	d	24	S		
7136	d	24	N		
7137	d	24	N		
7132	d	24	N		
7153	d	28	N		
7154	d	28	N		
7130	d	28	N		
7132	d	30	N		
7157*	k	42	T		Negative
7131*	d	60	T	Intestines	Hemangioma

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Table 31

Fate of Mice and Incidence of Neoplasm

Benzethonium - high level - males

Animal Number	Fate ⁽¹⁾	Weeks on test	Tissues ⁽²⁾ collected	Neoplasms observed	
				Gross (location)	Histologic (type)
7010	c	10	N		
7009	d	11	S		
7000	d	11	S		
7008	d	12	S		
7005	d	13	S		
7002	d	13	S		
6997	d	13	S		
6999	d	13	S		
6969	d	13	N		
6970	d	13	N		
6971	d	14	S		
7001	d	14	N		
13513	d	15	N		
7006	k	16	N		
6972	d	18	N		
6980	d	19	N		
6982	d	19	N		
6994	d	21	N		
6991	d	24	N		
13512	d	30	N		
13516	c	30	N		
6992*	k	32	T		Lymphomatous spleen
6989	k	33	T		
6990	d	33	N		
7003	c	35	N		
7004	d	43	N		
6998	d	46	N		
6993*	k	50	T		Negative
6984*	d	51	T		Negative
6976	d	59	N		
6996*	d	61	T		Negative

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Table 32

Fate of Mice and Incidence of Neoplasm

Benzethonium - high level - females

Animal Number	Fate (1)	Weeks on test	Tissues (2) collected	Neoplasms observed	
				Gross (location)	Histologic (type)
7047	d	5	N		
7036	d	13	S		
7019	d	13	S		
7048	d	16	N		
7062	d	17	N		
7046	d	17	N		
7058	d	18	N		
7027	d	20	N		
7023	d	22	N		
7021	d	24	N		
7057	d	27	N		
7054	d	29	N		
7039	d	29	N		
7059	d	31	N		
7034	d	37	N		
7016	c	37	N		
7013	d	45	N		
7028	d	45	N		
7044*	k	48	T		Negative
7015	d	49	N		
7025	c	51	N		
7022	d	52	N		
7024	d	53	N		
7031*	d	59	T		Negative
7026	d	59	T		Negative
7040	d	63	T	Lung	

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Table 33

Fate of Mice and Incidence of Neoplasm

Phenol Red - low level - males

Animal Number	Fate ⁽¹⁾	Weeks on test	Tissues ⁽²⁾ collected	Neoplasms observed	
				Gross (location)	Histologic (type)
6160	d	1	N		
6153	d	13	S		
6130	d	16	S		
6175	d	17	S		
6127	d	21	N		
6129	d	21	N		
6128	d	23	N		
6174	d	24	N		
6156	d	26	N		
6173	d	26	N		
6145	d	26	N		
6163	d	32	N		
6126	d	33	N		
6154	c	34	N		
6158	d	34	N		
6167*	d	42	T		Negative
6166	d	44	N		
6170*	d	44	T		Negative
6155	d	46	N		
6140*	d	49	T		Negative
6141*	d	49	T		Negative
6157*	d	49	T		Negative
6169*	d	50	T		Negative
6149	d	51	N		
6132	d	55	N		
6136*	d	55	T		Negative
6149	d	51	N		
6147	d	57	T		
6150*	d	59	T		Negative
6143	d	61	T		Negative
6135	d	65	T		
6137	d	60	T		

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Table 34

Fate of Mice and Incidence of Neoplasm

Phenol Red - low level - females

<u>Animal Number</u>	<u>Fate</u> ⁽¹⁾	<u>Weeks on test</u>	<u>Tissues</u> ⁽²⁾ <u>collected</u>	<u>Neoplasms observed</u>	
				<u>Gross (location)</u>	<u>Histologic (type)</u>
7308	d	7	S		
7284	d	9	S		
7268	d	10	N		
7291	d	13	S		
7265	d	15	S		
7264	d	18	N		
7274	d	18	N		
7267	d	21	N		
7287*	k	31	T		
7288	d	44	N		
7294	d	50	N		

Negative

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Table 35

Fate of Mice and Incidence of Neoplasm

Phenol Red - high level - males

Animal Number	Fate ⁽¹⁾	Weeks on test	Tissues ⁽²⁾ collected	Neoplasms observed	
				Gross (location)	Histologic (type)
7209	c	11	N		
7167	d	14	N		
7194	d	16	N		
7196	d	16	N		
7212	d	16	N		
7182	d	22	N		
7172	d	22	N		
7165	d	23	N		
7175	d	23	N		
7178	c	24	N		
7186	d	24	N		
7173	d	25	N		
7174	d	25	N		
7176	d	25	N		
7168	d	27	N		
7181	d	27	N		
7188	d	27	N		
7179	d	29	N		
7183	d	31	N		
7192	d	31	N		
7205*	d	50	T		
7171	d	59	N		
7211	d	56	T		

Table 36

Fate of Mice and Incidence of Neoplasm

Phenol Red - high level - females

Animal Number	Fate (1)	Weeks on test	Tissues collected (2)	Neoplasms observed	
				Gross (location)	Histologic (type)
7214	d	12	N		
7246	d	22	N		
7244*	d	30	T		Negative
7251	d	30	N		
7248	d	42	N		
7219*	d	44	T		Lymphomatous hyperplasia spleen
7221	d	48	N		
7241	d	48	N		
7238	k	49	T		
7261	d	56	N		
7279	d	60	N		
7252*	d	60	T		
7220*	d	61	T	Lung & uterus	Sarcoma; lung, liver, ovary

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Table 37

Fate of Mice and Incidence of Neoplasm

Thimerosal, N.F. - low level- males

Animal Number	Fate (1)	Weeks on test	Tissues collected (2)	Neoplasms observed	
				Gross (location)	Histologic (type)
6281	d	16	S		
6301	c	18	N		
6318	d	24	N		
6323	d	24	N		
6292	d	30	N		
6278*	d	32	T		Lymphomatous spleen
6293*	k	36	T		Lymphomatous spleen
6310	k	36	T		
6324	d	41	S		
6284	d	42	N		
6289	d	42	N		
6290*	d	42	T		Negative
6287	d	42	N		
6286	d	44	N		
6312*	d	46	T		Negative
6319	d	49	N		
6299*	d	53	N		Negative
6307	d	58	N		
11	d	59	T		
6322	d	59	T		Not neoplastic
6285*	d	62	T	Lung	Not neoplastic abscess

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Table 50

Fate of Mice and Incidence of Neoplasm

Thimerosal, N.F. - low level - females

(2)

Animal Number	Fate (1)	Weeks on test	Tissues collected	Neoplasms observed	
				Gross (location)	Histologic (type)
6339	k	7	N		
6340	k	10	N		
6331	d	15	N		
6361*	d	16	N		Negative
6334	d	18	N		
6359	d	20	N		
6337	d	25	S		
6373	d	39	N		
6346	d	40	N		
6335	d	44	N		
6327	d	44	N		
6348	d	44	N		
6333*	k	48	T	Subcutaneous	Subcutaneous abscess & granulation
6353	d	50	N		
6336*	d	58	T		Negative
6364	d	56	S	Uterus	Abscess - possibly necrotic & neoplasia
371	d	56	T		

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Table 39

Fate of Mice and Incidence of Neoplasm

Thimerosal, N.F. - High level - males

Animal Number	Fate (1)	Weeks on test	Tissues collected	(2) Neoplasms observed	
				Gross (location)	Histologic (type)
6214	d	12	S		
6199	d	12	N		
6183	d	13	S		
6190	d	13	N		
6202	d	13	S		
6212	d	13	S		
6188	d	14	N		
6186	d	15	N		
6182	d	18	N		
6208	d	41	N		
6210	d	41	N		
6205	d	42	N		
6206	d	43	N		
6207	d	43	N		
6196	d	48	N		
6218	d	48	N		
6219	d	48	N		
6177	d	49	N		
6181	d	53	N		
6179*	d	55	T		Negative

Negative

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Table 40

Fate of Mice and Incidence of Neoplasm

Thimerosal, N.F. - high level - females

Animal Number	Fate (1)	Weeks on test	Tissues collected (2)	Neoplasms observed	
				Gross (location)	Histologic (type)
6252	d	7	N		
6274	d	13	N		
6247	d	17	N		
6243	k	21	N		
6226	d	22	N		
6229	d	22	N		
6231*	d	32	T		Negative
6275*	d	35	T	Lung	Lung abscess
6233	k**	38	N		
6239	d	43	N		
6232*	k	56	T	Subcutaneous	Sarcoma (fibro)
6227	d	63	N		

* Examined at NIH

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Table 41

Fate of Mice and Incidence of Neoplasm

Methyl Parasept - low level - males

Animal Number	Fate (1)	Weeks on test	Tissues collected (2)	Neoplasms observed	
				Gross (location)	Histologic (type)
6506	d	7	N		
6507	d	11	S		
6477	d	21	S		
6490	d	23	N		
6496	d	26	N		
6486	d	27	N		
6475	d	28	N		
6482	c	28	N		
6488	d	31	N		
6499	d	32	N		
6491	d	38	N		
6494	d	42	N		
6501	k	42	N		
6498	d	44	N		
6504	d	45	N		
6502*	k	47	T		Early lung adenoma
6520	d	48	N		
6497	k	48	N		
6495	k	48	N		
6509*	d	49	T		Negative
6503*	d	51	T		Negative
6492	d	52	N		
6522*	k	54	T		Hyperplasia of spleen
6484*	d	62	T		Negative
6519	d	58	N		

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Fate of Mice and Incidence of Neoplasms

Methyl Parasept - low level - females

<u>Animal Number</u>	<u>Fate ⁽¹⁾</u>	<u>Weeks on test</u>	<u>Tissues collected ⁽²⁾</u>	<u>Neoplasms observed</u>	
				<u>Gross (location)</u>	<u>Histologic (type)</u>
6527	d	22	N		
6528	d	22	N		
6554	d	29	N		
6570	k	33	T		
6566*	k	35	T		Negative
6537	d	45	N		
6553*	k	58	T	Lung	No neoplasm on section
6569	d	58	T	Bladder	Negative

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Table 43

Fate of Mice and Incidence of Neoplasm

Methyl Parasept - high level - males

Animal Number	Fate (1)	Wks on test	Tissues collected (2)	Neoplasms observed	
				Gross (location)	Histologic (type)
6387	d	19	N		
6396	d	19	N		
6398	d	19	N		
6415	d	27	N		
6401	d	21	N		
6421	d	26	N		
6406	d	33	N		
6382	d	36	N		
6383	d	37	N		
6380	d	38	N		
6384*	d	44	T		Negative
6419	d	49	N		
6397	d	51	N		
6399*	k	51	T		Skin papilloma
6385	d	52	N		
6413	d	55	S	Lung neoplasm	Suppurative pneumonia
6395*	k	56	T		Skin papilloma with necrosis
6402*	d	59	T	Lung	Adenoma & suppurative pneumonia

Table 44

Fate of Mice and Incidence of Neoplasm

Methyl Parasept - high level - females

Animal Number	Fate (1)	Weeks on test	Tissues (2) collected	Neoplasms observed	
				Gross (location)	Histologic (type)
6461	d	19	N		
6439	d	25	N		
6448	d	29	N		
6436	d	30	N		
6442	k	31	N		
6443	k	31	N		
6444*	k	31	T		Negative
6445*	k	31	T		Negative
6446	d	31	N		
6447*	k	31	T		Negative
6425	d	32	N		
6457	d	33	N		
6464	d	33	N		
6426	c	34	N		
6449*	d	41	T		Negative
6429*	k	50	T		Negative
6433	d	45	N		
6454	d	58	N		
6472*	d	61	T		Negative
6450*	d	63	T	Uterus & liver	Sarcoma

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Table 45

Fate of Mice and Incidence of Neoplasm

Positive control - DBA in oil - low level - males

Animal Number	Fate (1)	Weeks on test	Tissues ⁽²⁾ collected	Neoplasms observed	
				Gross (location)	Histologic (type)
5763	k	12	N		
5730	k	18	S	Subcutaneous	Sarcoma
5745	k	19	S	Subcutaneous	Sarcoma
5752	k	19	S	Subcutaneous	Sarcoma
5735	k	21	N	Subcutaneous	
5742	d	21	N		
5747	d	21	N		
5765	k	21	S	Subcutaneous	
5748	d	23	N		
5743	d	24	S	Seminal vesic	Infection
5776	d	24	S		
5741	k	25	S	Subcutaneous	Sarcoma
5769	d	26	N		
5737	k	27	N	Subcutaneous	
5764	d	28	N		
5768	d	31	N		
5759*	k	33	T	Subcutaneous	Sarcoma
5750	d	34	N		
5773	k	34	N		
5734	d	37	N		
5766	d	39	N		
5744	d	39	N		
5738*	d	41	T		Negative
5736*	k	45	T		Lymphomatous spleen
5754*	k	51	T	Subcutaneous	
5770*	k	54	T		Negative

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Fate of Mice and Incidence of Neoplasm

Positive control - DBA in oil - low level - females

Animal Number	Fate (1)	Weeks on test	Tissues (2) collected	Neoplasms observed	
				Gross (location)	Histologic (type)
5786	d	7	N		
5823	d	13	N		
5822	k	18	S	Subcutaneous	Sarcoma
5813	k	19	S	Subcutaneous	
5804	k	19	S	Subcutaneous	Sarcoma
5803	d	19	N		
5800	d	19	N		
5779	d	19	N		
5789	d	20	S	- Subcutaneous	Sarcoma
5805	d	20	N		
5802	d	21	N		
5780	d	22	N		
5783	d	22	N		
5788	k	22	S	Subcutaneous	Sarcoma
5801	c	23	N		
5819	d	36	N		
5809*	k	43	T		Lymphosarcoma liver, spleen
5787	d	43	S	Subcutaneous	Sarcoma
5791	d	47	N		
5785	k	49	N		
5793	d	53	N		
5792*	d	54	T		Negative

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Table 47

Fate of Mice and Incidence of Neoplasm

Positive control - DBA in oil - high level - males

Animal Number	Fate (1)	Weeks on test	Tissues (2) collected	Neoplasms observed	
				Gross (location)	Histologic (type)
5643	k	15	S	Subcutaneous	Sarcoma
5647	k	16	S	Subcutaneous	Sarcoma
5667	k	20	S	Subcutaneous	Necrotic
5668	d	20	N		
5650	k	21	S	Subcutaneous	
5631	k	22	S	Subcutaneous	Sarcoma
5641	d	22	S	Subcutaneous	Sarcoma
5663	k	23	S	Subcutaneous	Sarcoma
5637	d	24	S		
5673	d	24	S	Subcutaneous	Sarcoma
5664	d	25	N		
5635	k	25	S	Subcutaneous	Sarcoma
5666	d	25	N		
5629	d	25	N		
5633	d	26	N		
5634	d	26	N		
549	k	28	S	Subcutaneous	Sarcoma
553	k	28	S	Subcutaneous	Sarcoma (abscess)
5655*	k	30	T	Subcutaneous	Sarcoma
5656*	k	30	T	Subcutaneous	Sarcoma
5636	d	31	N		
5640	d	31	N		
5638*	k	33	T	Subcutaneous	Sarcoma
5675	d	33	N		
5659	d	35	N		
5665*	d	35	N		
5662*	d	37	T		Negative
5658	k	37	T	Subcutaneous	Sarcoma
5660	d	39	N		
5661*	d	39	T		Negative
5652	d	40	T		
5654*	k	40	T	Subcutaneous	Sarcoma; cystic ovary
5630	d	42	S	Subcutaneous	Sarcoma
5674*	d	51	T		Negative
5642*	d	59	T		Papilloma, skin
5670	d	57	T		

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7-1-58 AR

Fate of Mice and Incidence of Neoplasm

Positive control - DBA in oil - high level - females

Animal Number	Fate ⁽¹⁾	Weeks on test	Tissues ⁽²⁾ collected	Neoplasms observed	
				Gross (location)	Histologic (type)
5725	k	13	S	Subcutaneous	
5693	k	15	S	Subcutaneous	
5684	k	16	S	Subcutaneous	Sarcoma
5685	k	17	S	Subcutaneous	Sarcoma
5686	d	17	S		
5687	k	17	S	Subcutaneous	Edematous salivary gland
5702	k	17	S	Subcutaneous	Sarcoma
5681	k	18	S	Subcutaneous	
5705	d	19	S	Mammary	Sarcoma
5726	k	19	S	Mammary	Sarcoma
5704	k	19	S	Mammary	Sarcoma
5694	d	22	N		
5703	d	22	S	Mammary	Sarcoma
5716	d	23	N		
5722	k	23	S	Subcutaneous	Sarcoma
5723	d	23	N		
5698	d	24	N		
5728	d	26	N		
5714	d	27	N		
5710	c	27	N		
5712	d	27	N		
5713	k	27	S	Subcutaneous	Sarcoma
5687	k	31	T	Subcutaneous	
5718*	k	31	T	Subcutaneous	Sarcoma, lymphomatous infiltration of lungs
5721	c	31	N		
5695	d	55	N		
5689*	d	61	T		Negative
5696	d	61	N		

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Table 49

Fate of Mice and Incidence of Neoplasm

Positive control - DBA in saline - low level - males

Animal Number	Fate (1)	Weeks on test	Tissues (2) collected	Neoplasms observed	
				Gross (location)	Histologic (type)
5268	d	9	N		
5292	d	9	S		
5290	d	10	N		
5293	d	10	S		
5255	d	11	N		
5289	d	12	N		
5257	d	15	N		
5258	d	20	N		
5272	d	21	N		
5256	d	24	N		
5294	d	26	S		
5263	d	28	N		
5261	d	29	N		
5284	d	31	N		
5281	k	32	N		
5282	k	32	T		
5279	d	36	N		
5	d	38	T		Negative
521/*	k	38	T		Lymphomatous spleen
5271	d	42	N		
5291	d	45	N		
5259*	k	45	T		Negative
5253	d	45	N		
5270	d	48	N		
5250*	k	51	T		Skin papilloma
5254*	d	53	N		Negative
5273*	d	54	T		Fibroma, urinary bladder, lymphomatous kidney
5260*	d	55	T		Skin papilloma with ne- crosis and abscess
5248*	d	57	T		Negative
5266	d	58	N		

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Fate of Mice and Incidence of Neoplasm

Positive control - DBA in saline - low level - females

Animal Number	Fate (1)	Weeks on test	Tissues (2) collected	Neoplasms observed	
				Gross (location)	Histologic (type)
5229	d	18	N		
5299	d	20	N		
5328	d	21	N		
5308	k	22	N		
5312*	d	28	T	Subcutaneous	Sarcoma
5310	d	31	N		
5295	k	33	T		
5313	c	35	N		
5314	d	35	N		
5298	d	39	N		
5317	d	42	N		
5320	d	46	N		
5307	d	49	N		
5296*	d	53	T	Lung	Lung abscess; not neo-
5297*	k	56	T		Negative plastic

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Table 51

Fate of Mice and Incidence of Neoplasm

Positive control - DBA in saline - high level - males

Animal Number	Fate (1)	Weeks on test	Tissues (2) collected	Neoplasms observed	
				Gross (location)	Histologic (type)
5388	d	8	N		
5360	d	9	N		
5343	d	10	N		
5366	d	10	S		
5387	d	13	N		
5341	d	13	S		
5355	d	15	N		
5383	d	20	N		
5386	d	20	N		
5385	d	21	N		
5349	d	21	N		
5348	d	22	N		
5389	d	23	N		
5382	d	29	N		
5384	k	31	N		
5350	d	33	N		
5340	d	37	N		
5376 *	d	38	T		Negative
5342 *	d	40	T		Negative
5361 *	d	50	T		Adenoma of lung
5364 *	d	52	T		Negative
5377 *	d	50	N		Lymphomatous infiltra
5346	d	55	N		of lung

Fate of Mice and Incidence of Neoplasm

Positive control - DBA in saline - high level - females

Animal Number	Fate ⁽¹⁾	Weeks on test	Tissues ⁽²⁾ collected	Neoplasms observed	
				Gross (location)	Histologic (type)
5415	d	5	S		
5416	d	8	N		
5426	d	24	N		
5425	d	25	N		
5436	d	39	N		
5390	d	45	N		
5419*	k	47	T		Negative

Table Explanation

Tables 52 through 90
Titled "Animals Sacrificed at Termination"

Table No.

Animals Sacrificed at Termination

Group Identification

<u>Animal Number</u> (1)	<u>Code</u> (2)	<u>Neoplasms Observed</u> (3)	
		<u>Gross (location)</u>	<u>Histologic (type)</u>

- (1) Animal number denotes the number assigned to each animal at the time they were weaned. Each weanling was identified by ear notches and assigned an identification number
- (2) Code - Indicates under what conditions histologic examination was made. At termination all surviving animals were sacrificed and 15 animals per sex per group were selected at random for complete histologic examination. These animals compose Code 1.

Where there were not 10 suitable animals from those dead on test to fulfill the number we wished to examine histologically from this group, the number necessary to make a total of 10 animals were chosen from animals sacrificed at termination. These animals make up Code 2.

If animals did not fall in Code 1 or Code 2 categories but had suspected tumors observed grossly, these suspect tissues were processed individually. These animals compose the Code 3.

(3) Neoplasms observed

Gross (location) Any enlargement or abnormality which could be suspected of being neoplastic on gross observation is noted as to location. A notation is entered for all animals and negative indicates no suspected neoplasm observed.

Histologic (type) Observations are noted with regard to neoplasms only and a notation made in all cases where histologic examination made whether a tumor was present or not.

Type of tumor is noted and also location if information not noted in "Gross (location)" column. If suspected gross tumor was shown by histology to not be neoplastic, this is noted and explained.

Table 53
Animals Sacrificed at Termination
Negative control - male

Animal No.	Code	Neoplasms Observed	
		Gross (location)	Histologic (type)
4849		Negative	
4855		Negative	
4856	1	Negative	Negative
4858	1	Mesenteric lymph node	Not neoplastic
4861	1	Negative	Negative
4866	1	Negative	Negative
4870		Negative	
4879		Negative	
4882		Negative	
4883	3	Injection site	Skin papilloma
4885	1	Negative	Negative
4886		Negative	
4888		Negative	
4891		Negative	
4895	1	Negative	Negative
4897	3	Lung	Adenoma
4898	1	Negative	Negative
4902		Negative	
4903		Negative	
4904		Negative	
4906		Negative	
4907	1	Negative	Negative
4908	1	Negative	Negative
4909		Negative	
4910		Negative	
4917		Negative	
4918		Negative	
4919	1	Negative	Negative
4922		Negative	
4923		Negative	
4928		Negative	
4929	1	Negative	Negative
4930		Negative	
4931		Negative	
4933	1	Negative	Negative
4934		Negative	
4935		Negative	
4936	1	Negative	Liver, adenomatous changes of biliary epithelium
4937		Negative	
4939		Negative	
4940		Negative	
4941	1	Testes	Not neoplastic
4942		Negative	
4943		Negative	
4944		Negative	

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Table 53 (continued)
Animals Sacrificed at Termination
Negative control - Males

Animal No.	Code	Neoplasms Observed	
		Gross (location)	Histologic (type)
4945	1	Negative	Negative
4953		Negative	
4957	1	Negative	Negative
4958	1	Negative	Negative
4959		Negative	
4961		Negative	
4962	1	Negative	Negative
4964	1	Negative	Negative
4965		Negative	
4966		Negative	
4967		Negative	
4968		Negative	
4969		Negative	
4975	1	Negative	Negative
4978	1	Negative	Negative
4979		Negative	
4980		Negative	
4981		Negative	
4982	1	Negative	Negative
4985		Negative	
4986		Negative	
4987	1	Negative	Negative
4990		Negative	
4991	1	Negative	Negative
4992	1	Negative	Negative
4993	3	Adrenal	Not neoplastic
4994		Negative	
4995		Negative	
4996		Negative	
4998	1	Negative	Negative
4999		Negative	
5003	1	Spleen	No neoplastic (hyperplasia
5004		Negative	
5012		Negative	
5014	1	Negative	Negative
5018		Negative	
5020		Negative	
5021		Negative	
5022		Negative	
5023		Negative	
5024		Negative	
5025		Negative	

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Table 53 (continued)

Animals Sacrificed at Termination

Negative control - males

Animal		Neoplasms Observed	
<u>No.</u>	<u>Code</u>	<u>Gross (location)</u>	<u>Histologic (type)</u>
5026	1	Spleen, kidney	Spleen, lymphomatous hyperplasia; kidney, small hemorrhagic cyst
5029		Negative	
5030		Negative	
5034	1	Negative	Negative
5035		Negative	
5036		Negative	
5038		Negative	
5039		Negative	

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Table 54
Animals Sacrificed at Termination
Negative control - female

Animal No.	Code	Neoplasms Observed	
		Gross (location)	Histologic (type)
5045	3	Lung	Adenoma
5048	3	Thymus, uterus	Thymus, lymphomatous; uterus, fibroma and cystic
5050		Negative	
5054		Negative	
5055		Negative	
5056	3	Lung	Adenoma
5057	1	Negative	Negative
5059		Negative	
5060	3	Thymus, ovary	Thymus, lymphosarcoma; ovary, hemangioma
5061		Negative	
5062	1	Negative	Negative
5063		Negative	
5064	1	Negative	Lung, adenoma
5066		Negative	
5068	1	Negative	
5071		Negative	
5075		Negative	
5076		Negative	
5077	3	Thymus	Not neoplastic
5078		Negative	
5079	3	Liver, spleen, pericardium	Sarcoma, undifferentiated at all sites, not neoplastic, suppurative metritis
5080	3	Uterus, ovary	
5081		Negative	
5082	1	Lung, mesenteric L.N.	Not neoplastic
5083	3	Urogenital tract	Not neoplastic, suppurative inflammation of the bladder
5085		Negative	
5087		Negative	
5089		Negative	
5092	1	Negative	Negative
5093		Negative	
5094		Negative	
5095		Negative	
5096	1	Negative	Lung adenoma
5097		Negative	
5098		Negative	
5100	1	Thymus, mesenteric L.N. pancreas	Lymphomatous changes
5102		Negative	
5103	1	Negative	Negative

Table 54 (continued)
Animals Sacrificed at Termination
Negative control - female

Animal No.	Code	Neoplasms Observed	
		Gross (location)	Histologic (type)
5104			
5105	3	Uterus	Not neoplastic (cystic endometritis)
5107	1	Ovary, adrenal	Not neoplastic (cystic ovary)
5108			
5109		Negative	
5110		Negative	
5111		Negative	
5112		Negative	
5113		Negative	
5114	3	Lung	Negative
5115		Negative	
5116	1	Lung	Adenoma
5119		Negative	
5121		Negative	
5122		Negative	
5123	1	Negative	Negative
5124		Negative	
5125		Negative	
5126		Negative	
5127		Negative	
5128		Negative	
5130		Negative	
5132	1	Negative	Negative
5133	1	Negative	Negative
5135		Negative	
5136		Negative	
5137		Negative	
5138		Negative	
5142		Negative	
5145		Negative	
5146		Negative	
5148		Negative	
5149		Negative	
5150	1	Negative	Negative
5151		Negative	
5152		Negative	
5153		Negative	
5154		Negative	
5155		Negative	

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Table 54 (continued)
Animals Sacrificed at Termination
Negative control - female

Animal No.	Code	Neoplasms Observed	
		Gross (location)	Histologic (type)
5156		Negative	
5157		Negative	
5159		Negative	
5160	3	Thymus	Lymphosarcoma
5162		Negative	
5163		Negative	
5164		Negative	
5165	1	Negative	Lymphomatous infiltration kidney, lung and mediastinal
5166		Negative	
5167	1	Negative	Negative
5168	3	Lung	Adenoma
5169	1	Negative	Negative
5170		Negative	
5171		Negative	
5172	1	Negative	Negative
5173	1	Negative	Negative
5174		Negative	
5175	1	Negative	Negative
5176	1	Negative	Lymphomatous kidney
5177	1	Negative	Negative
5178		Negative	
5179		Negative	
5180		Negative	
5181	1	Negative	Adenoma of lung
5182		Negative	
5183		Negative	
5184		Negative	
5185		Negative	
5186		Negative	
5189		Negative	
5191		Negative	
5192		Negative	
5193		Negative	
5197	1	Negative	Negative
5202		Negative	
5203		Negative	
5205	1	Negative	Negative
5206		Negative	

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Table 54 (continued)

Negative control - female

Animal No.	Code	Neoplasms Observed	
		Gross (location)	Histologic (type)
5208		Negative	
5210		Negative	
5215		Negative	
5217	3	Uterus	Not neoplastic, cystic metritis
5222		Negative	
5223	1	Lung	Adenoma
5225		Negative	
5226		Negative	
5227		Negative	
5228		Negative	
5229	1	Negative	Negative
5230		Negative	
5233	3	Fatty neoplasm thorax	Lymphosarcoma
5234	1	Lung	Adenoma
5235	1	Uterus	Negative
5236		Negative	
5237		Negative	
5238	1	Negative	Negative
5239		Negative	
5240		Negative	
5241	3	Lung	Adenoma
5242		Negative	
5243		Negative	
5244		Negative	

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Table 55
Animals Sacrificed at Termination
Pyridine - low level - males

Animal No.	Code	Neoplasms Observed	
		Gross (location)	Histologic (type)
5932	1	Negative	Negative
5935	1	Negative	Negative
5938	1	Lung	Not neoplastic, abscess
5940	1	Negative	Negative
5941	1	Negative	Negative
5942	2	Negative	Negative
5947	1	Negative	Negative
5948	1	Negative	Negative
5949	2	Negative	Negative
5950	1	Negative	Negative
5951	2	Negative	Negative
5952	1	Negative	Negative
5953	1	Negative	Negative
5954	2	Negative	Lung adenoma
5955	1	Negative	Negative
5956	2	Negative	Negative
5958	2	Negative	Negative
5959	1	Negative	Negative
5965	1	Negative	Negative
5967	2	Negative	Negative
5969	2	Negative	Negative
5972	1	Negative	Negative
5973	2	Negative	Negative
5978	1	Negative	Negative

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Table 56

Pyridine - low level - females

Animal No.	Code	Neoplasms Observed	
		Gross (location)	Histologic (type)
5984	1	Negative	Negative
5985	1	Negative	Negative
5986	1	Negative	Lymphomatous infiltration of lung & kidney
5987	1	Negative	Lymphomatous infiltration of lung & kidney
5988	2	Negative	Negative
5990	1	Uterus, liver	Uterus, fibrosarcoma; ovary, hemangioma; liver, normal
5991	1	Negative	Negative
5996	1	Uterus	Negative
5998	1	Uterus	Fibrosarcoma
5999	3	Uterus	Not neoplastic
6000	3	Uterus	Not neoplastic
6001	1	Negative	Negative
6002	2	Negative	Liver & kidney, lymphomatous infiltration
6008	1	Negative	Negative
6009	1	Thymus	Negative
6010	3	Uterus	Hemangioma
6011	3	Pancreas, uterus	Not neoplastic
6012	2	Negative	Negative
6013	1	Negative	Negative
6014	3	Ovary	Not neoplastic, cystic
6018	1	Negative	Negative
6019		Negative	
6020	2	Negative	Kidney, lymphomatous infiltration
6022	1	Negative	Negative
6023	1	Lung	Negative
6024		Negative	
6025	2	Negative	Negative
6026		Negative	
6028		Negative	

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Table 57
Animals Sacrificed at Termination
Pyridine - high level - males

Animal No.	Code	Neoplasms Observed	
		Gross (location)	Histologic (type)
5829	1	Negative	Lung adenoma
5830		Negative	
5832	2	Negative	Negative
5833	1	Negative	Negative
5834	1	Negative	Negative
5837	1	Negative	Negative
5939	2	Negative	Negative
5841	1	Negative	Negative
5842	3	Pancreas	Negative
5844	1	Negative	Negative
5846	1	Negative	Lung adenoma
5847	2	Negative	Negative
5848		Negative	
5850		Negative	
5854	2	Lung	Not neoplastic
5857	1	Negative	Negative
5858	1	Negative	Negative
5859	1	Negative	Negative
5862	1	Negative	Negative
5863	2	Negative	Pancreas and kidney, lymphomatous infiltration
5864	1	Negative	Negative
5865	2	Negative	Negative
5866	2	Negative	Negative
5871	1	Negative	Negative
5872	1	Negative	Negative
5873	2	Negative	Negative
5875	1	Negative	Negative
5876	2	Negative	Negative
5878	2	Negative	Negative

Table 58
Animals Sacrificed at Termination
Pyridine - high level - females

Animal No.	Code	Neoplasms Observed	
		Gross (location)	Histologic (type)
5879	2	Negative	Negative
5880	2	Negative	Negative
5881	2	Thymus	Kidney, lymphomatous infiltration
5882	2	Negative	Negative
5883	2	Negative	Negative
5885	1	Negative	Negative
5888	2	Negative	Sarcoma of lung
5889	1	Negative	Negative
5890	1	Negative	Kidney, lymphomatous infiltration
5892	1	Spleen, uterus	Uterus, fibrosarcoma; spleen, hyperplasi
5894	1	Negative	Negative
5895	1	Negative	Kidney, lymphomatous infiltration
5896		Negative	
5897	2	Negative	Negative
5898		Negative	
5899	3	Uterus	Not neoplastic
5900	3	Uterus	Mixed fibrosarcoma and fibroma
5901	1	Negative	Negative
5902	2	Negative	Negative
5903	3	Uterus	Not neoplastic
5906		Negative	
5907		Negative	
5908	1	Negative	Kidney, lymphomatous infiltration
5909	1	Negative	Negative
5910		Negative	
5911		Negative	
5912		Negative	
5914	3	Uterus	Not neoplastic
5915		Negative	
5916	1	Uterus	Uterus, not neoplastic; kidney, lymph- omatous infiltration
5917	1	Negative	Negative
5919	1	Negative	
5920		Negative	
5921		Negative	
5923	1	Uterus	Not neoplastic
5925		Negative	
5926		Negative	
5927	1	Negative	Negative
5928	1	Negative	Negative

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Table 59

Animals Sacrificed at Termination
Ethylene glycol - low level - male

Animal No.	Code	Neoplasms Observed	
		Gross (location)	Histologic (type)
6672	1	Negative	Negative
6674	1	Negative	Negative
6678		Negative	
6679	1	Negative	Negative
6680	1	Negative	Negative
6681	1	Negative	Negative
6683	1	Negative	Negative
6684		Negative	
6685	1	Negative	Negative
6687	1	Negative	Negative
6689	1	Negative	Negative
6690	1	Negative	Negative
6694		Negative	
6696	1	Negative	Negative
6697	1	Negative	Negative
6698		Negative	
6699	2	Negative	Negative
6700	2	Negative	Negative
6705		Negative	
6706	1	Negative	Negative
6707	1	Negative	Negative
6708		Negative	
6709	2	Negative	Negative
6710	2	Negative	Negative
6713		Negative	
6716		Negative	
6717	2	Negative	Negative
6718	2	Negative	Negative
6719	2	Negative	Negative
6720	1	Negative	Negative

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Animals Sacrificed at Termination
Ethylene glycol - low level - female

Animal No.	Code	Neoplasms Observed	
		Gross (location)	Histologic (type)
6721	1	Negative	Lymphomatous infiltration of kidney
6722	2	Negative	Negative
6726	2	Uterus	Not neoplastic, cystic endometritis
6727	2	Negative	Negative
6729		Negative	
6732	2	Negative	Negative
6733		Negative	
6734	1	Negative	Negative
6735	3	Ovary	Not neoplastic
6736	1	Negative	Negative
6737		Negative	
6738	1	Negative	Negative
6739	2	Negative	Negative
6740	1	Negative	Negative
6742		Uterus	
6743	1	Negative	Negative
6744	1	Uterus	Fibrosarcoma, uterus; lymphosarcoma, L.
6745		Negative	
6746	1	Negative	Liver & kidney (early lymphosarcoma)
6747	1	Negative	Lung, lymphoma
6748	1	Negative	Negative
6750	1	Negative	Negative
6751	1	Lung	Negative
6752		Negative	
6753		Negative	
6755		Negative	
6756	1	Negative	Lymphomatous infiltration of kidney
6757	1	Negative	Negative
6759		Negative	
6760		Negative	
6761		Negative	
6762		Negative	
6763	2	Negative	Negative
6764		Negative	
6765	3	Uterus	Not neoplastic, severe cystic endometritis
6768	1	Uterus	Not neoplastic, cystic endometritis

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Table 61
Animals Sacrificed at Termination
Ethylene glycol - high level - male

Animal No.	Code	Neoplasms Observed	
		Gross (location)	Histologic (type)
6031		Negative	
6034	1	Negative	Kidney, lymphosarcoma
6677		Negative	
6037		Negative	
6038	1	Negative	Negative
6040	2	Negative	Negative
6041	3	Lung	Adenoma
6042	1	Negative	Negative
6044	1	Negative	Negative
6047		Negative	
6049	2	Negative	Negative
6050	1	Negative	Negative
6052	2	Negative	Negative
6054	1	Negative	Negative
6055	1	Negative	Negative
6056	2	Negative	Negative
6058	1	Skin, postscapular L.N.	Not neoplastic; skin & lymph node show hyperplasia
6059	1	Negative	Negative
6062	1	Negative	Negative
6064	2	Negative	Negative
6066	1	Negative	Negative
6069	2	Negative	Negative
6070	1	Lung	No neoplasm or pathology in 3 lobes present on slide
6071	1	Negative	Negative
6072	1	Negative	Negative
6073	1	Negative	Negative

Worcester Alumni Research Foundation - Medical Research

Animals Sacrificed at Termination
Ethylene glycol -high level - female

Animal No.	Code	Neoplasms Observed	
		Gross (location)	Histologic (type)
6081	1	Uterus	Not neoplastic, cystic endometritis
6082	3	Uterus	Fibrosarcoma
6083	2	Negative	Negative
6086	2	Negative	Negative
6087		Negative	
6088	1	Negative	Negative
6089		Negative	
6091	1	Uterus	Not neoplastic, cystic endometritis
6092	2	Uterus	Not neoplastic, cystic endometritis
6093		Negative	
6096	1	Negative	Negative
6097	3	Uterus	Not neoplastic, cystic endometritis
6099	1	Spleen	Lymphosarcoma, spleen, liver, injection site
6100	1	Negative	Negative
6101	1	Negative	Negative
6103	3	Spleen, submaxillary L. N.	Lymphosarcoma
6104	2	Ovary	Not neoplastic, cystic
6111	1	Negative	Negative
6112		Negative	
6114	1	Negative	Fibrosarcoma, vagina, granuloma of lung
6115	1	Negative	Negative
6116			Negative
6117	1	Negative	Negative
6118	2	Negative	Negative
6119	1	Negative	Negative
6120	1	Negative	Negative
6121	1	Negative	Negative
6122		Negative	
6123		Negative	
6124		Negative	
6104			

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Table 63

Animals Sacrificed at Termination
2-chlorethanol - low level - male

Animal No.	Code	Neoplasms Observed	
		Gross (location)	Histologic (type)
6871	1	Negative	Negative
6872	1	Negative	Negative
6873	1	Negative	Negative
6874	1	Negative	Negative
6875	1	Negative	Lung adenoma
6876	2	Negative	Negative
6879	1	Negative	Negative
6881		Negative	
6883	1	Negative	Negative
6884	1	Negative	Negative
6886	1	Negative	Negative
6887	1	Negative	Negative
6890		Negative	
6891	1	Negative	Negative
6897		Negative	
6900	1	Negative	Negative
6801	1	Negative	Negative
6903	1	Negative	Negative
6905	1	Negative	Negative
6910		Negative	
6911		Negative	
6912		Negative	
6916		Negative	

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Animals Sacrificed at Termination
2-chlorethanol - low level - female

Animal No.	Code	Neoplasms Observed	
		Gross (location)	Histologic (type)
6919		Negative	
6920	1	Negative	Negative
6923	1	Lung	Not neoplastic, tissue metaplasia
6924		Negative	
6925		Negative	
6926	1	Negative	Negative
6927	1	Negative	Negative
6928		Negative	
6929	1	Negative	Negative
6932	1	Negative	Negative
6933	2	Negative	Negative
6934	1	Negative	Negative
6935			
6936	2	Skin	Not neoplastic
6937	2	Negative	Negative
6938	1	Negative	Negative
6939		Negative	
6940	3	Uterus	Not neoplastic, cystic endometritis
6941		Negative	
6942	1	Negative	Negative
6944		Negative	
6946	1	Negative	Negative
6946	1	Negative	Hepatitis
6947		Negative	
6949		Negative	
6950		Negative	
6952	2	Thymus, spleen	Splenic hyperplasia, not neoplastic
6953	1	Negative	Negative
6954	2	Negative	Negative
6955		Negative	
6956	1	Negative	Negative
6957		Negative	
6959	1	Negative	Negative
6960	1	Negative	Negative
6961		Negative	
6962	2	Negative	Negative
6963	1	Negative	Sarcoma (type ?) of submaxillary gland
6964	2	Negative	Negative
6965		Negative	
6966	2	Negative	Negative

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Table 65

Animals Sacrificed at Termination
2-chlorethanol - high level - male

Animal No.	Code	Neoplasms Observed	
		Gross (location)	Histologic (type)
6771		Negative	
6773	2	Negative	Negative
6774	1	Negative	Negative
6775		Negative	
6777	1	Negative	Negative
6781		Negative	
6782	1	Negative	Negative
6783		Negative	
6790	1	Negative	Negative
6791	1	Negative	Negative
6794	1	Negative	Negative
6795	1	Negative	Negative
6798		Negative	
6799	1	Negative	Negative
6801	2	Negative	Negative
6802	1	Negative	Negative
6803	1	Negative	Negative
6804	2	Negative	Negative
6805	1	Negative	Negative
6806	2	Negative	Negative
6807	2	Negative	Negative
6808	1	Negative	Negative
6810	2	Negative	Negative
6815	1	Negative	Negative
6816		Negative	
6817	1	Negative	Negative
6818	1	Negative	Negative
6820	2	Negative	Negative

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Animals Sacrificed at Termination
2-chlorethanol - high level - females

Animal No.	Code	Neoplasms Observed	
		Gross (location)	Histologic (type)
6821	1	Negative	Negative
6822		Negative	
6823		Negative	
6824		Negative	
6825	3	Uterus	Not neoplastic, cystic endometritis
6826	1	Negative	Hepatitis, lymphomatous infiltration in kidney
6827		Negative	
6828	1	Negative	Lymphomatous infiltration of kidney
6829	1	Negative	Negative
6831	1	Uterus	Lymphomatous infiltration of kidney; uterus not neoplastic (cystic)
6832	1	Spleen, uterus	Lymphosarcoma
6833	3	Uterus	Not neoplastic, cystic endometritis
6834		Negative	
6835	3	Skin	Not neoplastic, inflammatory
6836	2	Negative	Lymphomatous hyperplasia, thymus
6837	1	Negative	Hemangioma of ovary
6838		Negative	
6839	1	Negative	Adenoma of lung
6840	1	Negative	Negative
6841		Negative	
6842		Negative	
6843		Negative	
6844		Negative	
6845	3	Lung	Adenoma
6846		Negative	
6848		Negative	
6849	1	Negative	Negative
6850		Negative	
6851	1	Negative	Negative
6853		Negative	
6854		Negative	
6855		Negative	
6856	1	Negative	Negative

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Table 66 (continued)

Animals Sacrificed at Termination
2-chlorethanol - high level - females

Animal No.	Code	Neoplasms Observed	
		Gross (location)	Histologic (type)
6858	2	Uterus	Lymphomatous infiltration of kidney
6859	1	Negative	Negative
6861	3	Lung	Adenoma
6862	1	Negative	Ectopic bone in spleen, not neoplastic
6863		Negative	
6865		Negative	
6866		Negative	
6867	2	Negative	Lymphomatous infiltration of kidney
6868	1	Negative	Negative
6869	2	Negative	Negative
6770	2	Negative	Negative

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Table 67

Benzethonium - low level - male

Animal No.	Code	Neoplasms Observed	
		Gross (location)	Histologic (type)
7065	1	Negative	Negative
7066	3	Lung	Not neoplastic; abscess
7067	1	Negative	Negative
7069		Negative	
7070	1	Negative	Negative
7073	1	Negative	Negative
7074	1	Negative	Negative
7075	1	Negative	Negative
7076	1	Negative	Negative
7079	1	Negative	Negative
7080		Negative	
7081	2	Negative	Negative
7083	1	Negative	Negative
7084	1	Lung	Adenoma, necrotic
7087	1	Negative	Negative
7089		Negative	
7094			
7102	1	Negative	Negative
7103	1	Negative	Negative
7104	1	Negative	Negative
7105	1	Negative	Negative
7106		Negative	

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Table 68

Animals Sacrificed at Termination
Benzethonium - low level - females

Animal No.	Code	Neoplasms Observed	
		Gross (location)	Histologic (type)
7113		Negative	Negative
7114	1	Negative	Negative
7116	3	Uterus	Not malignant; cystic endometritis
7117	1	Negative	Negative
7118		Negative	
7119	2	Negative	Negative
7120	1	Negative	Negative
7121		Negative	
7122	2	Negative	Negative
7123		Negative	
7124	2	Negative	Negative
7125	1	Negative	Negative
7126	1	Negative	Negative
7127	2	Negative	Lymphosarcoma, kidney, mesenteric L
7129	1	Negative	Negative
7131			
7133		Negative	
7135	3	Pancreas	Not malignant - minimal lipomatosis
7138		Negative	
7139	3	Diaphragm, liver, pancreas	Pancreas & liver, cystic degeneration, focal, probably parasitic causes lymphomatous infiltration
7140	1	Negative	Negative
7141	2	Adrenal, ovary	Not neoplastic, kidney, lymphomatous infiltration
7142	2	Uterus	Not neoplastic, cystic endometritis
7143		Negative	
7144	1	Negative	Negative
7145		Negative	
7146	1	Negative	Negative
7147	1	Negative	Negative
7148		Negative	
7149	1	Negative	Negative
7151		Negative	
7152	1	Negative	Negative
7155	2	Negative	Negative
7156	1	Negative	Negative
7158	1	Pancreas	Adenoma of lung, pancreas not neoplas
7159	1	Negative	Negative
7161	2	Negative	Lymphomatous hyperplasia, spleen

Table 69
Animals Sacrificed at Termination
Benzethonium - high level - male

Animal		Neoplasms Observed	
No.	Code	Gross (location)	Histologic (type)
6968	2	Negative	Negative
6973	1	Negative	Negative
6974	1	Negative	Negative
6975	1	Negative	Negative
6977	1	Negative	Negative
6978	1	Negative	Negative
6979	1	Negative	Negative
6981	1	Negative	Negative
6983	2	Negative	Negative
6985	1	Negative	Negative
6986	1	Negative	Negative
6987	1	Negative	Negative
6988	1	Negative	Negative
6995	1	Negative	Negative
6996		Negative	
7007	2	Negative	Negative
7011	1	Negative	Negative
7012	1	Negative	Negative
13514	2	Kidney, liver	Not neoplastic; kidney, suppurative cystic nephritis, cystic hepatitis
13515	1	Negative	Negative

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Table 70

Animals Sacrificed at Termination
Benzethonium - high level - females

Animal No.	Code	Neoplasms Observed	
		Gross (location)	Histologic (type)
7015	1	Negative	Negative
7017	1	Negative	Negative
7018	1	Negative	Lung adenoma (ductile)
7020	2	Negative	Negative
7029	1	Negative	Negative
7030	1	Negative	Negative
7031		Negative	
7032	2	Negative	Negative
7033	2	Negative	Negative
7035	1	Negative	Negative
7037	1	Negative	Negative
7038	1	Negative	Negative
7041			
7042	1	Negative	Negative
7043	2	Negative	Negative
7045	2	Negative	Negative
7049	1	Lung	Adenoma of lung
7050		Negative	
7051	1	Uterus	Not neoplastic (cystic endometritis)
7052	1	Negative	Negative
7053	1	Negative	Lymphosarcoma
7055	1	Negative	Negative
7056	2	Negative	Negative
7060	1	Negative	Negative
7061	2	Negative	Negative

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Animals Sacrificed at Termination
Phenol red - low level - male

Animal No.	Code	Neoplasms Observed	
		Gross (location)	Histologic (type)
6131	1	Negative	Negative
6133	1	Negative	Negative
6134	1	Negative	Negative
6137		Negative	
6138	1	Negative	Negative
6139	1	Negative	Negative
6142	1	Negative	Negative
6144	1	Negative	Negative
6148	1	Negative	Negative
6149		Negative	
6151	1	Negative	Negative
6152	1	Negative	Negative
6161	1	Negative	Negative
6162	1	Negative	Negative
6164	1	Negative	Negative
6165	1	Negative	Negative
6171	1	Negative	Negative
6172			

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Table 72
Animals Sacrificed at Termination
Phenol red - low level - female

Animal No.	Code	Neoplasms Observed	
		Gross (location)	Histologic (type)
7263	2	Negative	Negative
7266	2	Negative	Negative
7669	1	Negative	Negative
7270	1	Negative	Negative
7271	3	Ovary, uterus	Not neoplastic; cystic endometritis, cystic ovary
7272	2	Negative	Negative
7273	3	Ovary	Not neoplastic, cystic
7275		Negative	
7276	1	Negative	Negative
7277	2	Uterus	Not neoplastic
7278	1	Lung, uterus	Lung, not neoplastic, chronic pneumonia; uterus, not neoplastic
7280	3	Submaxillary (subcu.)	Not neoplastic, abscess & granulomatous tissue
7281	1	Negative	Negative
7282	1	Negative	Negative
7283	2	Negative	Negative
7285	2	Negative	Kidney, lymphomatous infiltration
7286	2	Negative	Negative
7289	1	Negative	Negative
7290	2	Uterus	Not neoplastic, cystic endometritis
7292	1	Negative	Negative
7293	2	Negative	Negative
7295	1	Lung	Adenoma of lung
7296	1	Negative	Negative
7297		Negative	
7298	1	Negative	Negative
7299		Negative	
7300	1	Negative	Negative
7301		Negative	
7302	1	Negative	Negative
7303			
7304	1	Negative	Negative
7305		Negative	
7306		Negative	
7307		Negative	Negative
7308		Negative	
7309	1	Lung	No neoplasm, lymphoid nodule
7310		Negative	
7311		Negative	
7312	2	Negative	Negative

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Animals Sacrificed at Termination
Phenol red - high level - males

Animal No.	Code	Neoplasms Observed	
		Gross (location)	Histologic (type)
7163	1	Negative	Adenoma of lung
7164	1	Negative	Negative
7166	2	Negative	Negative
7169	2	Negative	Cystic kidney, papillomatous hyperplasia of pelvis or ureter
7170	1	Liver	Not neoplastic, focal necrosis
7177	1	Skin	Papillomatous hyperplasia of epithelium
7180	3	Heart, lung, spleen, pancreas	Sarcoma, undifferentiated, probably fibro.
7184	2	Negative	Cystic kidney
7185	1	Negative	Cystic kidney
7187	1	Negative	Negative
7189	1	Negative	Negative
7190	2	Negative	Negative
7191	2	Negative	Negative
7193	1	Negative	Negative
7195	1	Negative	Negative
7197	1	Negative	Negative
7198	1	Negative	Negative
7199	2	Negative	Negative
7200	2	Negative	Negative
7201		Negative	
7202	1	Negative	Negative
7203	1	Negative	Negative
7204	1	Negative	Negative
7206	2	Lung	No neoplasm on section
7207	2	Ureter	Not neoplastic, cystic
7208		Negative	
7210	1	Negative	Negative

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Table 74
Animals Sacrificed at Termination
Phenol red - high level - female

Animal No.	Code	Neoplasms Observed	
		Gross (location)	Histologic (type)
7213		Negative	
7215	3	Uterus	Fibrosarcoma
7216	1	Negative	Negative
7217	1	Negative	Negative
7218		Negative	
7219	2	Spleen	Lymphomatous spleen
7220	2	Ovary, liver, abdomen	Mixed fibro and lymphosarcoma ovary, liver and mesentary
7222		Negative	
7223	3	Uterus	Not neoplastic, cystic endometritis
7224		Negative	
7225	2	Negative	Negative
7226	1	Negative	Negative
7227	3	Ovary, pancreas, lung	Splenic nodule on pancreas; lung, not neoplastic; ovary normal
7228	3	Lung, spleen	Lymphosarcoma; lung, not neoplastic
7229	1	Negative	Negative
7230			
7231	1	Negative	Negative
7232		Negative	
7233	1	Negative	Negative
7234		Negative	
7235	1	Negative	Negative
7236		Negative	
7237		Negative	
7239	1	Negative	Negative
7240		Negative	
7242		Negative	
7243	2	Negative	Negative
7245	1	Negative	
7247	1	Negative	Adenoma of lung
7249	1	Negative	Negative
7250	2	Negative	Negative
7253	1	Negative	Negative
7254	2	Negative	Negative
7255	1	Pancreas, kidney, abdomen	Fibrosarcoma, pancreas and kidney possi mixed with lymphoma
7256	3	Pancreas	Necrotic fat with secondary granulation
7257		Negative	
7258	1	Negative	Lymphomatous kidney
7259	1	Lung	No neoplasm on sections presented
7260	2	Negative	Negative
7261	2	Negative	Negative

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Animals Sacrificed at Termination
Thimerosal, N.F - low level - male

Animal No.	Code	Neoplasms Observed	
		Gross (location)	Histologic (type)
6276	1	Lung	Not neoplastic, suppurative pneumonia
6277	1	Negative	Negative
6279		Negative	
6280			
6282	1	Negative	Negative
6283	1	Negative	Negative
6288		Negative	
6291	1	Lung, subcu. L.N., spleen	Not neoplastic, lung abscess, spleen and L.N. hyperplasia
6294	2	Negative	Negative
6295	1	Negative	Negative
6296		Negative	
6297		Negative	
6298	1	Negative	Negative
6300	2	Negative	Negative
6302		Negative	
6303	2	Negative	Negative
6304	1	Negative	Negative
6305	2	Negative	Negative
6306		Negative	
6308	3	Lung	Adenoma
6309		Negative	
6313	1	Negative	Negative
6314	1	Negative	Negative
6315	1	Negative	
6316	1	Negative	Negative
6317	1	Negative	Negative
6320	1	Spleen, bladder	Not neoplastic; chronic cystitis with granulation; spleen, hyperplasia
6321	1	Negative	Negative
6325	1	Negative	Negative

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Table 76

Animals Sacrificed at Termination
Thimerosal N. F. - low level - female

Animal No.	Code	Neoplasms Observed	
		Gross (location)	Histologic (type)
6326	3	Bladder	Not neoplastic
6328	1	Negative	Negative
6329	1	Bladder	Not neoplastic
6330	2	Negative	Negative
6332		Negative	
6338	2	Negative	Negative
6341	1	Negative	Negative
6342		Lung	Adenoma.
6343	2	Negative	Negative
6344	1	Negative	Negative
6345	1	Negative	Negative
6347	2	Uterus	Not neoplastic, cystic endometritis
6349	1	Negative	Negative
6350	2	Negative	Negative
6351		Negative	
6352	2	Negative	Negative
6353	2	Negative	Negative
6354			
6355		Negative	
6356		Negative	
6357		Negative	
6358	1	Negative	Lung adenoma
6360	1	Negative	Negative
6362		Negative	
6364	1	Negative	Negative
6365	2	Negative	Negative
6366		Negative	
6367	1	Negative	Negative
6368	2	Negative	Lymphomatous infiltration of kidney
6369	1	Negative	Negative
6370		Negative	
6372	1	Negative	Negative
6374	1	Negative	Negative

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Thimerosal N. F. - high level - male

Animal No.	Code	Neoplasms Observed	
		Gross (location)	Histologic (type)
6176	1	Negative	Negative
6178	1	Negative	Negative
6180	2	Negative	Negative
6182		Negative	
6184	1	Negative	Negative
6185	1	Negative	Negative
6187	2	Negative	Negative
6189	1	Negative	Negative
6191	1	Negative	Negative
6192	2	Negative	Negative
6193			
6194	1	Negative	Negative
6195	1	Negative	Negative
6197	1	Lung	Adenoma of lung
6198	1	Negative	Negative
6200	2	Lung	No neoplasm of lobe presented
6201	1	Negative	Negative
6203	1	Skin, base of skull	Papilloma
6204	2	Negative	Negative
6209	2	Negative	Negative
6211	2	Negative	Negative
6213	1	Negative	Negative
6215	1	Negative	Negative
6216	2	Negative	Negative
6217	1	Negative	Negative
6220	2	Negative	Negative
6221	3	Lung	Not neoplastic, chronic bronchiectasis
6222		Negative	
6223		Negative	
6224		Negative	
6225		Negative	

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Table 78

Animals Sacrificed at Termination
Thimerosal, N.F. - high level - female

Animal No.	Code	Neoplasms Observed	
		Gross (location)	Histologic (type)
6227			
6228		Negative	
6230		Negative	
6234	2	Negative	Negative
6235	2	Negative	Negative
6236	1	Lymphoid tissue enlarged	Lymphsarcoma, generalized
6237	2	Negative	Negative
6238		Negative	
6240		Negative	
6241		Negative	
6242	1	Negative	Negative
6244	1	Negative	Negative
6245		Negative	
6246	1	Negative	Negative
6248	1	Negative	Negative
6249	2	Negative	Negative
6250	2	Negative	Negative
6251	1	Negative	Negative
6253		Negative	
6254		Negative	
6255		Negative	
6256	3	Lung	Adenoma
6257	1	Negative	Negative
6258		Negative	
6259	2	Negative	Hemangioma of ovary
6260	1	Negative	Negative
6261	2	Negative	Negative
6262	1	Negative	Lung adenoma
6263	1	Negative	Lung adenoma
6264	1	Uterus, cervix	Fibrosarcoma
6265	1	Negative	Negative
6266	1	Negative	Negative
6267		Negative	
6268	1	Negative	Negative
6269	1	Negative	Negative
6270		Negative	
6271		Negative	
6272		Negative	
6273	3	Adrenal, ovary	Uterus, endometritis

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Animals Sacrificed at Termination
Methyl parasept - low level - male

Animal No.	Code	Neoplasms Observed	
		Gross (location)	Histologic (type)
6476	2	Negative	Negative
6478	1	Lungs	No neoplasm on 3 lobes presented
6479	1	Negative	Negative
6480	1	Negative	Negative
6481	1	Negative	Negative
6483	2	Negative	Negative
6484		Negative	
6485	1	Negative	Negative
6486	1	Negative	Negative
6489		Negative	
6493	1	Negative	Negative
6500	1	Negative	Negative
6505	2	Negative	Negative
6508	1	Negative	Negative
6510		Negative	
6511	2	Negative	Negative
6512	1	Negative	Negative
6513		Negative	
6514	2	Lung	Adenoma
6515	1	Negative	Adenoma of lung (early)
6516	1	Negative	Negative
6517		Negative	
6518		Negative	
6521	1	Negative	Adenomatosis area of lung
6523	1	Negative	Negative
6524	1	Negative	Negative

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Table 80

Animals Sacrificed at Termination
Methyl parasept - low level - females

Animal No.	Code	Neoplasms Observed	
		Gross (location)	Histologic (type)
6525	2	Uterus	Not neoplastic, cystic endometritis
6526	1	Negative	Negative
6529	1	Negative	Negative
6530	2	Negative	Negative
6531		Negative	
6532	1	Negative	Negative
6533	1	Negative	Negative
6534	2	Lung	Neoplasm not present on section
6535	2	Negative	Negative
6536	2	Negative	Negative
6538		Negative	
6539	1	Negative	Negative
6540	1	Negative	Negative
6541	1	Negative	Fibroma of uterus, lymphomatous infiltration of kidney
6542		Negative	
6543	2	Negative	Negative
6544	1	Negative	Negative
6545	1	Negative	Negative
6546	2	Negative	Negative
6547		Negative	
6548	1	Spleen	Lymphosarcoma
6549	1	Negative	Lymphomatous infiltration of kidney
6550		Negative	
6551	1	Negative	Lymphosarcoma
6552		Negative	
6555	3	Uterus	Not neoplastic, cystic endometritis
6556		Negative	
6557	2	Negative	Negative
6558		Negative	
6559		Negative	
6560		Negative	
6561	1	Negative	Negative
6562		Negative	
6563		Negative	
6564	1	Negative	Negative
6565		Negative	
6567		Negative	
6568		Negative	
6570	2	Negative	Negative

Table 87
 Animals Sacrificed at Termination
 Methyl parasept - high level - male

Animal No.	Code	Neoplasms Observed	
		Gross (location)	Histologic (type)
6375	1	Negative	Negative
6376	1	Negative	Negative
6377	1	Negative	Negative
6378		Negative	
6379		Negative	
6381		Negative	
6386	1	Negative	Negative
6388	2	Negative	Negative
6389		Negative	
6390		Negative	
6391	1	Negative	Negative
6392		Negative	
6393	2	Negative	Negative
6394	1	Negative	Negative
6400	1	Negative	Negative
6403	1	Negative	Negative
6404		Negative	
6405		Negative	
6407	1	Negative	Negative
6408	2	Negative	Negative
6409		Negative	
6410		Negative	
6411		Negative	
6412	2	Negative	Negative
6414	1	Negative	Negative
6416	1	Negative	Negative
6417	1	Negative	Negative
6418	1	Negative	Negative
6420		Negative	
6422	2	Negative	Negative
6423	1	Negative	Negative
6424	1	Negative	Negative

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Table 32
Animals Sacrificed at Termination
Methyl parasept - high level - female

Animal No.	Code	Neoplasms Observed	
		Gross (location)	Histologic (type)
6427	1	Negative	Negative
6428		Negative	
6430	2	Negative	Negative
6431		Negative	
6432	1	Negative	Negative
6434	2	Negative	Negative
6435	1	Negative	Negative
6437		Negative	
6438	1	Submaxillary	Not neoplastic, abscess
6440		Negative	
6441	3	Lung	Not neoplastic; abscess with granu- lamatous tissue
6451		Negative	
6452	2	Negative	Negative
6453	1	Negative	Lung adenoma
6454		Negative	
6455	1	Negative	Negative
6456	1	Negative	Negative
6458		Negative	
6459	3	Uterus	Not neoplastic, cystic endometritis
6460		Negative	
6462		Negative	
6463	1	Negative	Lung adenoma
6465	1	Negative	Negative
6466	1	Thymus	Not neoplastic
6467	1	Negative	Negative
6468	1	Negative	Negative
6469		Negative	
6471		Negative	
6472	1	Negative	Negative
6473	1	Negative	Negative
6474	1	Negative	Negative

Table 83
Animals Sacrificed at Termination
Positive control - DBA in oil - low level - male

Animal No.	Code	Neoplasms Observed	
		Gross (location)	Histologic (type)
5729	1	Negative	Negative
5731	1	Negative	Negative
5732	1	Lung	No neoplasm in 4 lobes presented
5733	2	Negative	Negative
5739	2	Negative	Negative
5740	1	Negative	Negative
5744	1	Negative	Negative
5749	1	Negative	Negative
5751	1	Negative	Negative
5755	1	Lung	Mixed fibrolymphosarcoma of urinary bladder; kypgm kinogisarcina
5756	1	Negative	Negative
5757	1	Negative	Negative
5758	1	Negative	Negative
5760	1	Negative	Cystic adenoma of lung
5761	1	Negative	Negative
5762	1	Negative	Negative
5767	2	Negative	Negative
5771	1	Negative	Negative
5772	2	Negative	Negative
5774	1	Negative	Negative
5775	1	Negative	Negative
5777	1	Negative	Negative
5778	1	Negative	Negative

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Table 84

Animals Sacrificed at Termination

Positive control - DBA in oil - low level - female

Animal No.	Code	Neoplasms Observed	
		Gross (location)	Histologic (type)
5781	2	Negative	Negative
5782	1	Negative	Kidney lymphomatous infiltration
5784	2	Negative	Negative
5790	1	Negative	Negative
5793	1	Kidney area	Not neoplastic, hemorrhagic
5794	2	Negative	Negative
5795	2	Negative	Negative
5796	2	Negative	Negative
5797	1	Negative	Negative
5798	1	Negative	Lung, early adenoma
5799	2	Negative	Negative
5806	1	Uterus	Negative
5807	1	Negative	Negative
5808	2	Liver, uterus, bladder	Sarcoma
5810	3	Lung	Not neoplastic, terminal hemorrhage
5811		Negative	
5812		Negative	
5814	1	Negative	Negative
5815		Negative	
5816		Negative	Negative
5817		Negative	
5818	1	Negative	Negative
5820	1	Negative	Negative
5821	1	Negative	Negative
5824	1	Negative	Negative
5825	1	Negative	Negative
5826		Negative	
5827	3	Site of injection (subcu.)	Sarcoma, probably spindle cell
5828	1	Negative	Negative

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Table 85

Animals Sacrificed at Termination

Positive control - DBA in oil - high level - male

Animal No.	Code	Neoplasms Observed	
		Gross (location)	Histologic (type)
5632	1	Negative	Negative
5639	1	Submaxillary	Not neoplastic
5644		Negative	
5645	1	Negative	Negative
5646	1	Negative	Negative
5648	1	Negative	Lung adenoma, hepatoma
5651	1	Negative	Negative
5657	1	Submaxillary, lung	
5666	1	Negative	Negative
5669	1	Negative	Negative
5671	1	Lung	Adenoma
5672	1	Negative	Negative
5676	1	Negative	Negative
5677	1	Negative	Negative
5678	1	Mesenteric L.N.	Not neoplastic, hyperplasia

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Table 86

Animals Sacrificed at Termination

Positive control - DBA in oil - high level - females

Animal No.	Code	Neoplasms Observed	
		Gross (location)	Histologic (type)
5679		Negative	
5680	1	Lung	Adenoma
5682	1	Negative	Negative
5683	1	Negative	Negative
5688	1	Negative	Negative
5690	2	Negative	Negative
5691	2	Bladder and/or colon	Negative
5692	1	Negative	Negative
5697	2	Ovary	Not neoplastic, hemorrhagic cyst
5699	1	Negative	Negative
5700	1	Lung	Adenoma
5701	1	Negative	Negative
5707	2	Negative	Lung adenoma
5708	1	Negative	Negative
5709	1	Negative	Negative
5710	1	Negative	Negative
5711	1	Negative	Negative
5715	2	Negative	Subcu., Necrosis, probably a sarcoma; lymphomatous spleen
5717	1	Negative	Negative
5720	1	Negative	Adenoma of lung
5724	2	Lung	No neoplasm in 4 lobe sections presented
5727	2	Negative	Negative

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Table 87

Animals Sacrificed at Termination

Positive control - DGA in saline - low level - male

Animal No.	Code	Neoplasms Observed	
		Gross (location)	Histologic (type)
5245	1	Negative	Negative
5246		Negative	
5247	1	Negative	Negative
5249	1	Negative	Negative
5251	1	Negative	Negative
5252	1	Negative	Negative
5262	1	Negative	Negative
5264			
5265	1	Negative	Negative
5267	1	Negative	Negative
5269	2	Negative	Negative
5274	2	Negative	Negative
5276	1	Negative	Negative
5278	1	Negative	Negative
5280	1	Spleen	Lymphomatous hyperplasia
5283	3	Subcutaneous	Abscess
5285	1	Lung	Not neoplastic, chronic pneumonia
5286	1	Lung	Not neoplastic, chronic pneumonia
5287	1	Negative	Negative
5288	1	Negative	Negative

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Table 88

Animals Sacrificed at Termination

Positive control - DBA in saline - low level - females

Animal No.	Code	Neoplasms Observed	
		Gross (location)	Histologic (type)
5300	3	Spleen	Not neoplastic
5301	1	Negative	Negative
5302		Negative	
5303	2	Pancreas	Negative
5304		Negative	
5305	1	Negative	Negative
5306	1	Lung	Adenoma
5309	1	Negative	Negative
5311		Negative	
5315		Negative	
5316		Negative	
5318	2	Negative	Negative
5319	1	Negative	Negative
5321	1	Lung (small)	Thyroid, adenoma; lung, no neoplasm on se
5322	1	Negative	Negative
5323	2	Negative	Negative
5324		Negative	
5325	1	Negative	Lung adenoma
5326	1	Negative	Negative
5327	2	Negative	Negative
5330	1	Negative	Negative
5331	1	Negative	Negative
5332	1	Negative	Negative
5333	1	Uterus	Not neoplastic, cystic endometritis
5334		Negative	
5335	1	Negative	Negative
5337	2	Negative	Negative
5338	2	Negative	Negative
5339	1	Negative	Lymphomatous infiltration of kidney

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Table 89

Positive control - DBA in saline - high level - males

Animal No.	Code	Neoplasms Observed	
		Gross (location)	Histologic (type)
5344		Negative	
5345	1	Negative	Negative
5347	1	Negative	Negative
5351	1	Lung	Adenoma
5352	1	Negative	Lung adenoma
5353	1	Lung	Adenoma
5354	1	Negative	Pancreas, sarcoma, mixed lympho and fibro
5356	1	Lung	Adenoma
5357	1	Negative	Lung adenoma
5358	2	Negative	Negative
5359	1	Lung	No neoplasm on 3 lobes presented
5362	1	Negative	Negative
5363		Negative	
5365	2	Negative	Negative
5367	1	Negative	Negative
5368	2	Lung	Negative
5369	3	Lung	Adenoma, early
5370	2	Negative	Negative
5371	2	Negative	Negative
5372	3	Lung	No neoplasm in sections presented
5373	1	Negative	Lung adenoma (early)
5374	1	Lung	Adenoma, necrotic
5375	1	Base of head, subcu.	Possibly lymphosarcoma, advanced necrosis
5378	1	Lung	Adenoma
5379		Negative	
5380	3	Lung	Adenoma
5381	3	Lung	No neoplasm on 3 lobes presented

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Table 90

Animals Sacrificed at Termination

Positive control - DBA in saline - high level - female

<u>Animal No.</u>	<u>Code</u>	<u>Neoplasms Observed</u>	
		<u>Gross (location)</u>	<u>Histologic (type)</u>
5391	1	Lung	No neoplasm in 4 lobes presented
5392	2	Negative	Negative
5393	1	Negative	Lung adenoma
5394	2	Negative	Negative
5395	1	Lung	Lymphosarcoma, mediastinal lymph node; lung adenoma
5396	1	Lung, spleen	Lymphosarcoma of pancreas and liver; adenoma of lung
5397		Negative	
5398		Negative	
5399		Negative	
5400	2	Negative	Negative
5401	1	Negative	Negative
5402	1	Thymus	Lymphosarcoma
5404		Negative	
5405	1	Thorax	Lymphosarcoma
5406	1	Uterus	Uterus not neoplastic; lymphosarcoma spleen and thorax
5407	2	Ovary, omentum	No neoplasm in sections observed
5408	3	Lung	Adenoma
5409	3	Lung	Sarcoma with bony metaplasia
5410	1	Lung, uterus	Lung adenoma, uterus not neoplastic
5411	2	Lung	Adenoma
5412	1	Negative	Negative
5413		Negative	
5414		Negative	
5417	3	Lung	Adenoma
5418	3	Lung	Adenoma
5420	1	Lung	Adenoma
5421	2	Negative	
5422	3	Uterus	Not neoplastic, cystic endometritis
5423		Negative	
5424	3	Lung	No neoplasm in 3 lobes presented
5427		Negative	
5428	3	Lung	Adenoma
5429	2	Negative	Lung adenoma, early
5430	1	Lung	Adenoma
5431	2	Negative	Cystic and lymphomatous kidney
5432	1	Lung, uterus	Lung adenoma, uterus not neoplastic
5433	2	Lung	No neoplasm in 3 lobes presented
5434	1	Lung	Negative
5435		Negative	
5437	1	Uterus	Not neoplastic

TOXICOLOGY AND CARCINOGENICITY OF PRESERVATIVES USED IN THE PREPARATION OF BIOLOGICAL PRODUCTS

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Abstract

The toxic and carcinogenic potential of seven preservatives and extracting agents used in biological products was studied. The compounds were benzethonium chloride, ethylene chlorohydrin, ethylene glycol, thimerosal, methyl paraben, phenol red, and pyridine.

Groups of newborn Swiss mice, weanling strain A/HeJ mice and weanling Fischer 344 rats were injected with each of the compounds either as single or multiple doses. None of the compounds were found to be carcinogenic for the mice. Six of the seven caused no adverse reactions in Fischer rats. One, benzethonium chloride, was implicated in the development of subcutaneous fibrosarcomas at the injection sites in 26 of the 200 rats given this compound. No metastases developed. In each group of 200 control animals or animals injected with other compounds, only 2-4 fibrosarcomas developed. Nickel sulfate was injected into a group of Fischer 344 rats and over 90 % developed sarcomas at the injection sites. These sarcomas were more pleomorphic, resembled rhabdomyosarcomas and metastasized.

Benzethonium chloride should be considered a relatively weak carcinogen. Nevertheless, other preservatives shown to be non-carcinogenic should be substituted.

This report is based on the ideas and work of many persons,* and I shall act as the mere spokesman. The studies extended over a number of years but were essentially coordinated through the Collaborative Program of the Division of Biologics Standards (DBS), now the Bureau of Biologics, FDA. In 1967, under the direction and planning of Dr C. W. Hiatt, the rapporteur of this session, the DBS began studies concerning the toxicity and potential carcinogenicity of certain chemicals used in the preparation of biological products. The seven compounds investigated were: benzethonium chloride, thimerosal, methyl paraben, phenol red, ethylene glycol, ethylene chlorohydrin and pyridine. The first three are preservatives and it is only to these that we shall turn our attention in this report. Some of the findings were published previously by Mason *et al.* (7).

Studies were undertaken in several strains of mice, and in one inbred strain of rats. Table I lists the animals used. Preliminary studies were carried out in order to determine the level of each chemical that induced 100 % mortality in the animals as well as those

* Collaborators listed in Appendix A.

Table I. *Animals inoculated with preservatives*

Species and strain of rodent	Sex	Age at first inoculation
Mouse, Swiss, general purpose	Male, female	Newborn
Mouse, Swiss, general purpose	Male, female	4 weeks
Mouse, Balb/c	Male, female	4 weeks
Mouse, A/He Jax	Male, female	12 weeks
Mouse, CF-1	Female	Newborn
Mouse, C57BL/6	Male	12 weeks
Rat, Fischer 344	Male, female	4 weeks

Table II. *Rats inoculated with preservatives.*
Toxicological data from preliminary studies

Compound	Single injection			Repeated injections MTD ^a (mg/kg)	Maximum dose selected for chronic study
	LD ₅₀ (mg/kg)	95 % confidence limits	LD _{0.1} (mg/kg)		
Benzethonium choride	119.0	67-211	39	3.0	3.0
Thimerosal	98.0	82-117	26	5.0	1.0
Methyl paraben	500.0	—	—	—	3.5

^a Maximum tolerated dose.

levels which caused 50 % mortality (LD₅₀). The maximum tolerated doses of each substance were also determined for animals other than newborns. An example of the type of data found in these preliminary studies in rats is shown in Table II.

The final studies varied according to the laboratory doing the study, the strain of animal used and the age of the animals inoculated. This is summarized in Table III. Thus, newborn Swiss mice were inoculated only once subcutaneously and the observation period was to be 15 months. This procedure followed that outlined by Kelly *et al.* (6), used to study known carcinogens in newborn mice and modeled after the methods used to investigate the oncogenesis of viruses (4). Weanling Swiss and Balb/c mice were to be given multiple injections either subcutaneously or intraperitoneally every two weeks for 20 injections. However, because of difficulties, only 15 to 16 injections were given and most of the animals were observed for 18 months. A similar group of mice was given a single inoculation of each chemical as controls.

Mice of strains A/He Jax and CF-1 were inoculated intravenously once only. These mice develop adenomas of the lungs after injection of any known carcinogens and this model is a particularly sensitive indicator of carcinogenesis (8). One group of CF-1 mice was injected intravenously once monthly for seven months. At the time of sacrifice, seven months, the number of lung adenomas seen on the surface was compared to those seen in control animals and in animals given dibenzpyrene.

Groups of Fischer 344 rats were injected subcutaneously twice weekly for one year with various doses of each chemical. Animals were sacrificed at 12 or 18 months.

In all the studies, groups of control animals were injected with saline. In several of the studies, a group of uninjected animals was also kept throughout the observation period.

at first inoculation
newborn
weeks
weeks
weeks
newborn
weeks
weeks

Maximum dose selected for chronic study (g)
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Table III. *Experimental design*

Species and strain of rodent	Sex	Age at first inoculation	Number of inoculations	Route of inoculation
Mouse, Swiss, general purpose	Male, female	Newborn	Single	Subcutaneous
Mouse, Swiss, general purpose	Male, female	4 weeks	Single; multiple	Subcutaneous, intraperitoneal
Mouse, Balb/c	Male, female	4 weeks	Single; multiple	Subcutaneous, intraperitoneal
Mouse, A/He Jax	Male, female	12 weeks	Single	Intravenous
Mouse, CF-1	Female	Newborn	Multiple	Intravenous
Mouse, C57BL/6	Male	12 weeks	Multiple	Subcutaneous
Rat, Fischer 344	Male, female	4 weeks	Single; multiple	Subcutaneous

Dibenzpyrene and 1,2,5,6-dibenzanthracene were used as carcinogen controls in mice, and nickel sulfide in rats. The chemicals under test were given at two or more dose levels.

Gross autopsies were performed on all the animals in some studies and on a percentage of the normal appearing animals in others. Histologic examination of representative tissues was done as appropriate, either when gross observations indicated abnormalities or on a certain percentage of all the animals.

Toxicity of the compounds was indicated by mortality, weight of the animals throughout the observation period and organ abnormalities. At the dosages used (below the lethal level), even when the chemicals were given repeatedly, little or no toxicity was noted. However, in the initial experiments performed to establish toxic dose levels, it was noted that when benzethonium chloride (70 mg/kg) was injected subcutaneously in the C57BL/6 mice (having black fur), spotty depigmentation of the fur occurred about 34 days later. The majority of animals injected with 35 mg/kg of this compound also had a similar depigmentation. Some animals developed ulcerations of the skin shortly after inoculation but these healed relatively rapidly. Histologic examination of injection sites in mice as well as rats showed similar findings for all chemicals injected. There were multiple granulomas with numerous cysts lined by endothelial-like cells and giant cells. Some of the cysts were filled with amorphous material containing crystalline clefts. These granulomas were surrounded by proliferating fibroblastic tissue. Thus benzethonium chloride appeared to be an irritative material in these mice and, as will be noted below, this is of significance concerning this particular chemical.

No other significant evidence of toxicity was noted in regard to these chemicals. However, in several studies, a number of animals, both in the control groups and the animals under test, developed intercurrent infections, particularly pneumonia. Some of these died but others recovered. The major significance of this was that fewer healthy animals thus were available for final evaluation of the effect of these chemicals. However, the total number of animals was felt to be sufficient.

The results of the long-term studies in mice, both newborn and weanling, did not indicate that a significant number of tumors occurred in test animals as compared to controls. As was expected, in both controls and test animals a number of different tumors did occur. Of particular interest are the results of the intravenous injection of these chemicals into the strains A/He J and CF-1 mice, since this model is considered particularly suitable for carcinogenesis studies (Table IV). As can be seen, the number of mice of either strain as well as the number of tumors were not significantly different from those in animals injected with saline. However, mice injected with a known carcinogen developed significantly more adenomas of the lung. Thus, using this model, none of the three preservatives studied caused any evidence of carcinogenicity in mice.

The results of the study in Fischer rats were somewhat different(7). This study involved the repeated injection of the compounds subcutaneously twice weekly for one year. As shown in Table V, 200 animals were injected with each compound. The highest dose chosen was the maximum tolerated dose established in previous studies. The dose level was adjusted on a mg/kg basis so that although 0.25 ml was the mean volume injected, this was varied in order to achieve the proper dosage. The dose levels for each compound, except methyl paraben, were half-log₁₀ intervals. Since methyl paraben is insoluble, a saturated solution was used as the highest dose level and the other levels were set out at quarter-

in controls in mice, or more dose levels, and on a percentage basis of representative observed abnormalities

the animals throughout (below the lethal toxicity was noted. At these dose levels, it was noted usually in the C57BL/6 about 34 days later. It also had a similar effect after inoculation at sites in mice as there were multiple giant cells. Some of the cells had clefts. These effects of benzethonium will be noted below,

to these chemicals. In control groups and particularly pneumococcal, the significance of this evaluation of the results was felt to be

in the handling, did not use animals as controls. In animals a number of results of the intraperitoneal CF-1 mice, since in these studies (Table I) as the number of animals injected with each dose developed significantly the three preserva-

different (7). This was done by subcutaneously twice injected with each dose established on a basis so that all animals in order to achieve the same effect of methyl paraben, saturated solution set out at quarter-

Table IV. Lung adenomas in mice 28 weeks after intravenous inoculation

Compound	Dose/mouse (mg)	Strain A/He J			Strain CF-1		
		% with adenomas	Average no. adenomas/mouse		% with adenomas	Average no. adenomas/mouse	
Benzethonium chloride	0.35	25.6	1.1	19.5	1.1	23	1.3
Methyl paraben	2.5	14.0	1.3	21.7	1.4	20	1.2
Thimerosal	0.2	3.3	1.2	10.2	1.0	5.3	1.0
Saline (0.2 ml)	—	14.0	1.0	14.5	1.0	11.1	1.0
Dibenzopyrene	0.05	68.1	8.7	34.5	3.6	—	—
	0.1	83.3	10.8	85.7	11.2	—	—
	0.5	—	—	81.4	3.8	—	—

Table V. *Chronic study dose levels^a in Fischer 344 rats (administered subcutaneously twice weekly for one year)*

Compound	Dose (mg/kg)	No. animals started
Benzethonium chloride	3.0	80
	1.0	60
	0.3	40
	0.1	20
Thimerosal (merthiolate)	1.0	80
	0.3	60
	0.1	40
	0.03	—
Methyl paraben	3.5	80
	2.0	60
	1.1	40
	0.6	20
Vehicle control (saline)	0.25 ml	120
Negative control	None	120

^a After 52 weeks of treatment the animals were kept for observation for an additional six months.

Table VI. *Nickel sulfide inoculation of rats*

Route of inoculation	Dose (mg)	No. of animals
SC	10.0	40 ^a
SC	3.3	40
IM	10.0	40
IM	3.3	40

^a 20 males, 20 females.

log₁₀ intervals. Animals were observed for six months beyond the inoculation period. Males and females in equal numbers were used. Control rats (60 males, 60 females) were used as listed in Table V. In addition, rats were given a single subcutaneous or intramuscular injection of nickel sulfide (Ni₃S₂) as shown in Table VI. This compound has been shown to induce sarcomas in rats by a number of investigators (5, 9).

Injection site tumors were observed as shown in Table VII. The number of animals listed in the denominators indicates those that survived either for the total observation period of 18 months, or were sacrificed because of large local tumor at 12 months, or when moribund with tumor. As can be seen, the known carcinogen induced injection site tumors in 92% of animals inoculated. Only in animals injected with one of the compounds under study, namely benzethonium chloride, was there any significant evidence of injection site tumors (13%).

None (r
Saline (r
Methyl
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Ni₃S₂

Compound
Benzethonium cl

Ni₃S₂

These tumors which grew ste tumors was dos reactions. Tabl occurred after response wher which were no the sarcomas i sembled rhabd before other sp the injection s pleomorphic a evidence of me irritation and Grasso & Go Grasso & Go carcinogen.

Table VII. Incidence of injection site tumors in rats

Compound ^a	No. of animals	% of animals
None (negative controls)	1/100	1
Saline (vehicle controls)	0/100	0
Methyl paraben	3/200	1.5
Thimerosal	4/200	2
Benzethonium chloride	25/200	13
Ni ₃ S ₂	146/158	92

^a All dose levels included.

Table VIII. Incidence of injection site tumors in rats

Compound	Dose	No. of animals	Injection site tumors		Other tumors	
			No.	%	No.	%
Benzethonium chloride	3.0	80	10	12.5	8	10
	1.0	60	8	13.3	5	8.3
	0.3	40	2	5	4	10
	0.1	20	0	0	1	5
Ni ₃ S ₂	10 ^a	40	37	92.5	—	—
	3.3 ^b	39	37	95	—	—
	10 ^c	40	34	85	—	—
	3.3 ^c	40	38	95	—	—

^a mg/kg SC.^b mg total SC.^c mg total IM.

These tumors were fibrosarcomas which had little tendency to metastasize but which grew steadily to a large size. In this group of rats, the development of the tumors was dose-related and correlated with the high incidence of granulomatous reactions. Table VIII shows the dose-response relationship of the tumors which occurred after benzethonium chloride injections in contrast to the lack of dose-response when Ni₃S₂ was given to the animals. Furthermore, other tumors which were noted in the animals were not dose-related. It should be noted that the sarcomas induced by Ni₃S₂ were highly pleomorphic and malignant, resembled rhabdomyosarcomas and metastasized widely. Thus, these animals died before other spontaneous tumors could occur. In contrast, the tumors related to the injection site in animals inoculated with benzethonium chloride were less pleomorphic and less malignant. Furthermore, in only one animal was there evidence of metastasis. This type of fibrosarcoma occurring at the site of repeated irritation and granulomatous inflammation has been described previously by Grasso & Golberg(1) and others(3). Using the classification proposed by Grasso & Golberg(2), benzethonium chloride should be considered a weak carcinogen.

In summary, the toxic and carcinogenic potential of these compounds was studied. Only one, benzethonium chloride, was implicated in the development of injection-site fibrosarcomas which were of a low level of malignancy. Thus benzethonium chloride should be considered a relatively weak carcinogen. Nevertheless, other preservatives shown to be non-carcinogenic should be substituted.

REFERENCES

- (1) GRASSO, P. & GOLBERG, L. (1966). Early changes at the site of repeated subcutaneous injection of food colorings. *Food Cosmetic Toxicology* 4, 269-82.
- (2) GRASSO, P. & GOLBERG, L. (1966). Subcutaneous sarcoma as an index of carcinogenic potency. *Food Cosmetic Toxicology* 4, 297-320.
- (3) GRICE, H. C. & MANNELL, W. A. (1966). Rhabdomyosarcomas induced in rats by intramuscular injections of Blue VRS. *Journal of the National Cancer Institute* 37, 845.
- (4) GROSS, L. (1956). Viral (egg-borne) etiology of mouse leukemia. Filtered extracts from leukemia C58 mice, causing leukemia (or parotid tumors) after inoculation into newborn C57 Brown of C3H mice. *Cancer* 9, 778-91.
- (5) HUEPER, W. C. (1952). Experimental studies in metal carcinogenesis: 1. Nickel cancer in rats. *Texas Report in Biology and Medicine* 10, 167-86.
- (6) KELLY, M. G. & O'GARA, R. W. (1961). Induction of tumors in newborn mice with Dibenz [a, h] anthracene and 3-Methylcholanthrene. *Journal of the National Cancer Institute* 26, 651-79.
- (7) MASON, M. C., CATE, C. C. & BAKER, J. (1971). Toxicology and carcinogenesis of various chemicals used in the preparation of vaccines. *Clinical Toxicology* 4, 185-204.
- (8) STEWART, H. L. (1959). Pulmonary tumors in mice. In: *The Physiopathology of Cancer*, pp. 18-37, ed. F. Homburger. London: Hoeber and Harper & Row.
- (9) SUNDERMANN, F. W. & SUNDERMANN, F. W., Jr. (1961). Nickel poisoning. XI. Implications of nickel as a pulmonary carcinogen in tobacco smoke. *American Journal of Clinical Pathology* 35, 203-9.

APPENDIX A

Collaborators

DBS: C. W. Hiatt, S. H. Singer, C. E. Miller, E. A. Fitzgerald.
 Mason Research Institute: M. M. Mason, C. C. Cate, J. Baker.
 Bio-Research Consultants, Inc.: F. Homburger.
 WARD Institute: P. Derse, P. O. Nees.

Discussion

M. FIELD (Discussant) Mr Chairman, I wish to make several comments about this most interesting work of Dr Kirschstein and her colleagues at FDA.

The first point relates to the results obtained by intravenous inoculation of each of the three preservatives into mice. On the basis of the data presented, Dr Kirschstein has concluded that none of the preservatives studied caused any evidence of carcinogenicity in mice. Yet, her data show that injection of benzethonium chloride into the strain A/He J mouse gave 25.6% adenomas versus 14% for the saline controls. With the CF-1 strain of mouse, a single injection of preservative gave 19.5% adenomas for benzethonium chloride and 21.7% for methyl paraben. This contrasts with 14.5% in the controls. These differences were even greater when these two preservatives were administered to the

CF-1 strain of for methyl par

At first glar paraben may b mentioned: Fi Kirschstein's d for the rat inoc no information the conclusions especially since potential.

A second ite incidence of sp saline. Assumi like to know w of diagnosis be about tumors h Such informati Again, we have relation to how histopathology clusions of her

The high inc suggest a possi whether these electron micros chemical carcin

A last point ethonium chloi carcinogenic ac observed in the istered in high little, if any, us reason is that t negative micro- 'what is the st commonly used disinfectants, a contact with hu carcinogenesis of action to the that recent evic induce tumors v the nucleus. If solution forms ammonium com manifested on t cosmetic lotion?

Thank you.

KIRSCHSTEIN A performed much well. It is the on Unfortunately, I

CF-1 strain of mouse in seven monthly doses (i.e. 23% for benzethonium chloride, 20% for methyl paraben and 11.1% for the saline controls).

At first glance, it would seem to me that both benzethonium chloride and methyl paraben may be weak carcinogens in these strains of mice. However, two points should be mentioned: First, the total number of mice for each group was not indicated in Dr Kirschstein's data. Are we to assume that the same numbers of animals were used here as for the rat inoculation groups (i.e. 100 controls and 200 test animals per group)? Secondly, no information was presented in the way of statistical evaluation to support or contradict the conclusions presented. It seems to me that statistical analysis should be included here, especially since it is obvious that these chemicals may have low or marginal carcinogenic potential.

A second item of interest regarding the A/He J and CF-1 mouse data is the very high incidence of spontaneous lung adenomas observed in the control groups inoculated with saline. Assuming that the lung adenomas were diagnosed by gross observation, I would like to know whether there was sufficient histopathological evidence to show consistency of diagnosis between the controls and the test animals. In other words, are we talking about tumors having the same cellular characteristics in the controls as the test animals? Such information could very well affect the overall percentages reported in the data. Again, we have here the question of how many animals were observed with adenomas in relation to how many were studied by histopathology. Dr Kirschstein indicated that histopathology was performed on some groups of animals, but I do not recall any conclusions of her findings.

The high incidence of lung adenomas in the control CF-1 and A/He J mice might also suggest a possible viral association with these lesions. It would be of interest to know whether these tumors are associated with C particles or other virus-like particles by electron microscopic examination. It would be interesting to know, for example, whether chemical carcinogens in mice might activate a latent virus in this particular host.

A last point that I would make is that I fully agree with Dr Kirschstein that benzethonium chloride, within the experimental conditions reported here, does have low carcinogenic activity for the Fischer 344 strain of rat. The injection site fibrosarcomas observed in these animals appear to be significant along with the toxicity when administered in high doses. To my knowledge, benzethonium chloride at the present time has little, if any, usage as a preservative in biological vaccines, serums, toxoids, etc. The reason is that this compound and other quaternaries have little effect against Gram-negative micro-organisms. Perhaps a greater question that is raised by this information is 'what is the status of this compound and other quaternary ammonium compounds commonly used in large amounts in many cosmetic preparations, household cleaners, disinfectants, and the like?' Some of these materials may have frequent or even daily contact with human skin or mucous membranes for many years. Is there any danger of carcinogenesis under these conditions? Although it is impossible to assign a mechanism of action to the carcinogenic activity of benzethonium chloride at this time, we do know that recent evidence reveals that many nitrogen-containing electrophilic carcinogens induce tumors via the mechanism of direct or indirect alkylation of nucleic acids within the nucleus. If such a mechanism can be assigned to benzethonium chloride, which in solution forms an electrophilic radical, then would we not expect other quaternary ammonium compounds to have similar carcinogenic activity, and would this activity be manifested on the skin, for example, through long-term exposure to some antibacterial cosmetic lotion?

Thank you.

KIRSCHSTEIN All the studies reported were carried out under contract, and some were performed much better than others. The rat study was the one that was done extremely well. It is the one we have the most data on, and we therefore spent the most time on it. Unfortunately, I cannot tell how many mice of the strain A, or of strain CF-1 were used

compounds was the development of malignancy. Thus weak carcinogenic should be

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lation of each of the Dr Kirschstein has of carcinogenicity in to the strain A/He J with the CF-1 strain s for benzethonium the controls. These administered to the

in each case. However, the investigator who has had a vast experience with the lung adenoma system did not feel that the data showed any statistical difference in the occurrence of adenomas in the two strains of animals. In addition, the histopathology did not differ. The tumors in animals inoculated with the preservatives did not differ from those in the saline, or the dibenzpyrene controls.

Electron microscopy was not done on these animals. However, it had been done on many of the other systems worked on at the National Cancer Institute. I believe that in most any mouse one can find C-type particles and, as you probably know, Dr Huebner believes now that all chemical carcinogenesis is simply activation of viral particles.

I think other quaternary ammonium compounds should be studied. At the time we undertook the study, benzethonium chloride was present in inactivated poliomyelitis vaccine and DPT-polio vaccine. It was considered to be the preservative of choice for the quadruple vaccine because of its lack of deleterious effect on the pertussis component. I am sure that those still interested in that product would be very reluctant to use any other preservative.

Finally, the question of injection-site tumors in rats is open to considerable debate. Some say that this does not indicate that the injected materials are really carcinogenic. Other than in DPT-polio vaccine in Canada, benzethonium chloride is not used for the comparable product in the US. The only product that I know, in which it is used, is topical thrombin, and that would seem to be less significant.

FIELD To set the records straight, I believe that benzethonium chloride was considered to be detrimental to the pertussis component. It was because thimerosal was found destructive to poliovirus that benzethonium chloride was added to the triple vaccine.

MENGEL I have a question about the toxic dose of phenol in man. I think of snake venom or botulinus antisera, for example. Persons receiving these get between 100 and 500 milligrams of phenol. Are these quantities tolerated by man?

BENJAMINI Your question I assume refers to toxicity, rather than hypersensitization or carcinogenicity. It is an important question to know what happens to the recipients of these amounts of phenol.

MÖLLER With regard to the possibility of polluting the human body, it was apparently demonstrated that acute and chronic toxicity of these substances was very low. However, does anyone know what is the fate of these substances when introduced in the body? Has Dr Kirschstein or anyone else studied the distribution in tissues of radioactive-tagged thimerosal or parabens? Mercurial compounds, for example, are said to be concentrated in kidney tissue.

KIRSCHSTEIN I have no information in this respect.

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Toxicology and Carcinogenesis of Various Chemicals Used in the Preparation of Vaccines*

Marcus M. Mason, D.V.M., C. C. Cate, and John Baker

*Mason Research Institute
Worcester, Massachusetts*

INTRODUCTION

The objective of this study was to determine the toxic and carcinogenic potential of the following seven compounds, commonly used as preservatives or extracting agents in the preparation of commercially available biologics:

Merthiolate (Thimerosal), benzethonium chloride, methyl paraben, phenol red, pyridine, ethylene glycol, and ethylene chlorohydrin.

The investigation was divided into three stages.

1. Acute toxicity and an approximation of the LD₅₀.
2. A four-week injection period at five dose levels to determine the maximum tolerated dose.
3. A long-term (1 year) inoculation series (twice weekly), subcutaneously into rats at four dose levels with careful evaluation of the incidence of tumors.

*This work was performed under Contract Number Ph43-67-676 for the Division of Biologics Standards, NIH.

MATERIALS AND METHODS

Animals

Caesarean-derived, Fischer 344 weanling (4-weeks-old) rats of both sexes were used in this project. The animals weighed approximately 60 gm on arrival and were placed on test within two weeks after arrival. They were housed two to a cage in air-conditioned quarters and were maintained on Purina Rat Chow and water *ad libitum*. The animal quarters were divided into four separate rooms all on one floor. Approximately 2300 rats were used in this trial with 1800 continuing on long-term study.

Chemicals

The compounds were provided by the Division of Biologic Standards, National Institutes of Health. A list of the compounds is provided in Table 1.

Experimental Procedure. The project was begun in three stages.

1. An initial toxicologic study to determine the acute lethal dose (LD_{50}).
2. A supplementary study to determine the maximum tolerated dose.
3. The final four-dose-level study to determine carcinogenicity of the compounds in animals treated for at least one year and held another year for observation.

The compounds were prepared in solution with sterile physiological saline (Cutter Saftiflask "28" sodium chloride, injection, U.S.P., N.S.S.), and administered subcutaneously, twice weekly, for the required period. The syringes used were individually packaged, sterile, nonpyrogenic, 1 cc disposable tuberculin syringes (TOMAC Catalog No. 15085 25 D) with a 25 X 5/8 needle size. The volume of compound injected was adjusted according to animal weight so that the mean injection volume for each compound approximated 0.25 ml during the chronic study, except where the solubilities necessitated a larger dose volume. Dosages of the compounds were administered on a mg/kg basis. A fixed volume of 0.25 ml per injection of saline was used in the vehicle control group.

Formulation

All compounds, with one exception (phenol red), were formulated from the commercial product every two weeks and only used for the two-week

TOXIC AND CARCINOGENIC CHEMICALS

Table 1
Toxicological Data from Preliminary Studies

Compound	Single injection		Repeated injections, MTD, mg/kg	Maximum dose selected for chronic study
	LD_{50} , mg/kg	95% confidence limits		
Benzethonium chloride	119.0	67-211	3.0	3.0
Ethylene chlorohydrin	71.6	-	< 30.0	10.0
Ethylene glycol	5300.0	3857-7478	< 1700.0	1000.0

MATERIALS AND METHODS

344 weanling (4-weeks-old) rats of both sexes were used. The animals weighed approximately 60 gm on arrival. They were housed in stainless steel cages within two weeks after arrival. They were conditioned quarters and were maintained on *ad libitum*. The animal quarters were divided into one floor. Approximately 2300 rats were continuing on long-term study.

Studies were conducted by the Division of Biologic Standards, U.S. Department of Health, Education and Welfare. A list of the compounds is provided in Table 1.

The project was begun in three stages. The first stage was to determine the acute lethal dose.

The second stage was to determine the maximum tolerated dose. The third stage was to determine carcinogenicity of the compounds treated for at least one year and held for observation.

The compounds were prepared in solution with sterile physiological saline solution (0.9% sodium chloride, injection, U.S.P., N.S.S.), and were injected, twice weekly, for the required period. The compounds were aseptically packaged, sterile, nonpyrogenic, 1 cc (TOMAC Catalog No. 15085 25 D) with a volume of compound injected was adjusted so that the mean injection volume for each compound was 0.25 ml during the chronic study, except where a larger dose volume. Dosages of the compounds were on a mg/kg basis. A fixed volume of 0.25 ml was used in the vehicle control group.

Compounds (phenol red), were formulated from the beginning of the study and only used for the two-week

Table 1
Toxicological Data from Preliminary Studies

Compound	Single injection		Repeated injections, MTD, mg/kg	Maximum dose selected for chronic study
	LD ₅₀ , mg/kg	95% confidence limits		
Benzethonium chloride	119.0	67-211	3.0	3.0
Ethylene chlorohydrin	71.6	-	< 30.0	10.0
Ethylene glycol	5300.0	3857-7478	< 1700.0	1000.0
Thimerosal	98.0	82-117	< 5.0	1.0
Methyl paraben	> 500.0	-	-	3.5
Phenol red	> 600.0	-	-	1.0
Pyridine	866.0	649-1155	< 180.0	100.0

period. A stock solution of 600 mg/liter of phenol red was prepared every six weeks, from which the biweekly dilutions were made.

Initial Toxicity Study (Single Dose)

Five dose levels, at half-log intervals, were administered to groups of approximately 20 animals each, with equal numbers of males and females. The middle dose level was selected on the basis of literature data to closely approximate the LD₅₀. Lethality information calculated from the results was used to determine the starting dose in the supplementary study.

Supplementary Study

Sixty animals were separated into five groups of 6, 12, 24, 12, and 6 animals each, with equal numbers of males and females, and administered injections of compounds twice weekly for four weeks at dose levels separated by quarter- or half-log intervals. The information from these results was used to determine the maximum tolerated dose level to be used in the long-term study. Table 1 summarizes the data from the first two studies.

Chronic Study

The final study was initiated within three months after the start of the contract. Two hundred animals were used for each compound tested. These were divided into four groups containing 80, 60, 40, and 20 rats (with equal numbers of males and females in each group), to be treated with four dose levels, high to low, respectively. The dose levels for each compound, except for methyl paraben and phenol red, were separated by half-log intervals. The insolubility of methyl paraben and phenol red prevented the establishment of the MTD. Therefore, a saturated solution was used as the highest dose level, and other levels were set at quarter-log intervals. The treatment consisted of twice-weekly injections for 52 weeks, with the dose level maintained on the mg/kg basis by adjusting the injection volumes to the animal weights. All animals were weighed weekly throughout the study. Animals from the preliminary studies that were already on dose levels selected for the chronic study were used to supplement the corresponding groups in the chronic study. Table 2 shows the doses used for each compound.

Table 2
Chronic Study Dose
(administered subcutaneously twice weekly)

Compound	Dose, mg/kg
Benzethonium chloride	3.0
	1.0
	0.3
	0.1
Ethylene chlorohydrin	10.0
	3.0
	1.0
	0.3
Ethylene glycol	1000.0
	300.0
	100.0
	30.0
Thimerosal (merthiolate)	1.0
	0.3
	0.1
	0.03
Methyl paraben	3.5
	2.0
	1.1
	0.6
Phenol red	1.0
	0.56
	0.32
	0.18
Pyridine	100.0
	30.0
	10.0
	3.0
Ni ₃ S ₂	10.0 mg s.
Positive control	3.3 mg s.
(single injection)	10.0 mg i.
	3.3 mg i.
Vehicle control (saline)	0.25 ml
Negative control	None

^a After 52 weeks of drug treatment the :
for an additional six months.

d within three months after the start of the study. The dose levels used for each compound tested. The groups containing 80, 60, 40, and 20 rats and females in each group), to be treated low, respectively. The dose levels for each paraben and phenol red, were separated by the toxicity of methyl paraben and phenol red from the MTD. Therefore, a saturated solution level, and other levels were set at quarter-levels. The treatment consisted of twice-weekly injections for 52 weeks. The animals were maintained on the mg/kg basis by adjusting the dose levels to animal weights. All animals were weighed weekly. The animals from the preliminary studies that were selected for the chronic study were used to determine the dose levels in the chronic study. Table 2 shows the results of the study.

^aAfter 52 weeks of drug treatment the animals were kept for observation for an additional six months.

Control

Three types of controls were used: (a) vehicle controls (60 males, 60 females) received twice weekly injections of saline at 0.25 ml per injection. (b) negative controls (60 males, 60 females) received no treatment; and (b) positive controls (80 males, 80 females) received predetermined fixed doses of nickel sulfide (Ni_3S_2). The positive controls were divided into four subgroups: A, B, C, and D of 40 animals each (20 males, 20 females). Each animal in the A and B groups received a single subcutaneous injection of 10.0 and 3.3 mg, respectively. Groups C and D were given the same doses of nickel sulfide by a single intramuscular injection in the thigh. The powder was suspended in Duracilin A.S. (sterile procaine penicillin G, aqueous suspension, 300,000 units/cc), and administered in 0.1 ml volumes.

Data and Records

Animals were examined each day by the technicians and all abnormalities were reported immediately. A weekly record was kept of animal weights, injection volumes, and gross observations. All experimental animals were necropsied after they died or were sacrificed. Organ weights were obtained and selected tissues preserved for histopathologic study.

Histopathology

Each animal on test was autopsied either at 12 months or at 18 months as planned. All spontaneous deaths, moribund animals, and those showing gross pathology or abnormal organ weights were examined histologically in addition to those chosen for routine examination.

RESULTS

Toxicity

Only three major criteria were considered for assaying toxicity. These are:

1. Survival time.
2. Weight gains.
3. Drug-related organ pathology.

Survival Time. The first criterion is met in Table 3 which details the monthly and cumulative rat mortality. It clearly shows that for the first

TOXIC AND CARCINOGENIC CHEMICALS IN

Table 3
Cumulative Rat Mo

Treatment group	Total number started	12 m
		Total mortality
Benzethonium chloride	200	3
Ethylene chlorohydrin	200	4
Ethylene glycol	200	4
Thimerosal	200	3
Methyl paraben	200	5
Phenol red	200	4
Pyridine	200	3
Controls		
Negative	120	4
(no treatment)		
Vehicle (saline)	120	4
Positive (Ni_3S_2)	160	120

nine months there was little mortality in any group (where deaths were principally due to rapid tumor growth). For all other groups was less than 1% for this period. For the 12-month treatment period the mortality was still less than 2.0%. It was during the last five months that the mortality in the negative and vehicle controls the range varied between 4.0% and 9.0%. In test-drug groups it varied from 4.0% to 9.0% with 9.0%, while benzethonium chloride and ethylene glycol had a mortality of 7.5%. There was a fairly even distribution except for the bronchopneumonia seen in the details later).

Weight Gains. Weekly weight determination and the results of these body weight observations are given in Table 5 for the 12-month period and in Table 5 for the 18-month period. For the 12-month period it would appear that the treatment groups caused any retardation of weight gains compared to the untreated and vehicle controls. Benzethonium chloride caused a retardation of weight gain of 14% (6-21%) (5-16%) while Thimerosal at its highest dose showed

used: (a) vehicle controls (60 males, 60 injections of saline at 0.25 ml per injection; 60 females) received no treatment; and 50 females) received predetermined fixed. The positive controls were divided into of 40 animals each (20 males, 20 females). ps received a single subcutaneous injection. Groups C and D were given the same e intramuscular injection in the thigh. uracilin A.S. (sterile procaine penicillin G, ts/cc), and administered in 0.1 ml volumes.

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RESULTS

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Table 3
Cumulative Rat Mortality

Treatment group	Total number started	12 months		18 months	
		Total mortality	% mortality	Total mortality	% mortality
Benzethonium chloride	200	3	1.5	15	7.5
Ethylene chlorohydrin	200	4	2.0	15	7.5
Ethylene glycol	200	4	2.0	10	5.0
Thimerosal	200	3	1.5	18	9.0
Methyl paraben	200	5	2.5	13	6.5
Phenol red	200	4	2.0	12	6.0
Pyridine	200	3	1.5	8	4.0
Controls					
Negative (no treatment)	120	4	2.0	7	5.8
Vehicle (saline)	120	4	2.0	10	8.3
Positive (Ni ₃ S ₂)	160	120	75.0	144	90.0

nine months there was little mortality in any group but the positive control (where deaths were principally due to rapid tumor growth). The mortality for all other groups was less than 1% for this period. By the end of the 12-month treatment period the mortality was still very low ranging from 1.5 to 2.0%. It was during the last five months that the mortality increased. In the negative and vehicle controls the range varied between 5.8% to 8.3% while in test-drug groups it varied from 4.0% to 9.0%. Thimerosal was highest with 9.0%, while benzethonium chloride and ethylene chlorohydrin both had a mortality of 7.5%. There was a fairly even distribution of deaths except for the bronchopneumonia seen in the Thimerosal-treated rats (fuller details later).

Weight Gains. Weekly weight determinations were made of each animal and the results of these body weight observations are summarized in Table 4 for the 12-month period and in Table 5 for the results at the end of 18 months. For the 12-month period it would appear that only three compounds caused any retardation of weight gains as compared to the untreated and vehicle controls. Benzethonium chloride has an average retardation of weight gained of 14% (6-21%). Pyridine averaged 11% (5-16%) while Thimerosal at its highest dose showed a decrease of 10%

Table 4
Comparative Mean Weights of the Highest Dosage Group of Test Compounds
End of Treatment (12 months)

Compound	Approx. mean age, weeks	Weeks on test	Mean wt., gm				Final body wt., % control			
			No. of rats	Male	No. of rats	Female	Neg. σ control	Vehicle control	Neg. ϕ control	Vehicle control
Negative control	59	53	49	406	50	247		96		94
Vehicle control	58	53	46	423	50	262	104		106	
Benzethonium chloride	57	51	40	333	40	233	82	79	94	89
Ethylene chlorohydrin	58	52	30	454	28	265	112	107	107	101
Ethylene glycol	59	53	30	416	29	259	102	98	105	99
Thimerosal	57	53	30	365	28	234	90	86	95	89
Methyl paraben	57	51	40	423	38	264	104	100	107	101
Phenol red	60	53	30	443	30	272	109	105	110	104
Pyridine	59	53	29	357	28	234	88	84	95	89

Table 5
Comparative Mean Weights of the Highest Dosage Group of Test Compound
18 Months after Start of Treatment

Compound	Approx. mean age, weeks	Weeks on test	Mean wt., gm				Final body wt., % control			
			No. of rats	Male	No. of rats	Female	Neg. σ control	Vehicle control	Neg. ϕ control	Vehicle control
Negative control	84	78	47	430	46	310		101		96
Vehicle control	84	78	46	426	44	322	99		104	
Benzethonium chloride	84	78	19	372	19	281	87	87	91	87

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TOXIC AND CARCINOGENIC CHEMICALS II

control	59	53	49	406	50	247		96		94
Vehicle control	58	53	46	423	50	262	104		106	
Benzethonium chloride	57	51	40	333	40	233	82	79	94	89
Ethylene chlorohydrin	58	52	30	454	28	265	112	107	107	101
Ethylene glycol	59	53	30	416	29	259	102	98	105	99
Thimerosal	57	53	30	365	28	234	90	86	95	89
Methyl paraben	57	51	40	423	38	264	104	100	107	101
Phenol red	60	53	30	443	30	272	109	105	110	104
Pyridine	59	53	29	357	28	234	88	84	95	89

Table 5
Comparative Mean Weights of the Highest Dosage Group of Test Compound
18 Months after Start of Treatment

Compound	Approx. mean age, weeks	Weeks on test	Mean wt., gm.				Final body wt., % control				
			No. of rats			No. of rats	Female	Neg. ♂ Vehicle		Neg. ♀ Vehicle	
				Male				control	control	control	control
Negative control	84	78	47	430	46	310		101		96	
Vehicle control	84	78	46	426	44	322	99		104		
Benzethonium chloride	84	78	19	372	19	281	87	87	91	87	
Ethylene chlorohydrin	84	78	29	439	26	331	102	103	107	103	
Ethylene glycol	84	78	28	434	28	324	101	102	105	101	
Thimerosal	84	78	26	331	25	249	77	78	80	77	
Methyl paraben	84	78	29	432	27	314	100	101	101	98	
Phenol red	84	78	30	421	27	305	98	99	98	95	
Pyridine	84	78	29	420	28	304	98	99	98	94	

(5-14%). These figures were compiled only for the highest dose levels of all compounds. At lower doses the retardation of weight gains were less significant.

The 18-month compilation shown in Table 5 shows that there was a good recovery almost to normal by the animals in the pyridine group but that the weight gains by the Thimerosal and benzethonium groups were still retarded. The Thimerosal-treated rats were the most affected with weight retardation of 22% (20-23%), while those receiving benzethonium chloride recovered slightly so that at the end of the experiment there was a weight lowering of 12% (9-13%).

Drug-Related Organ Pathology. During the examination of about 2000 rats, a great variety of pathology was observed. The most frequent of these were mild changes in the liver, kidneys, heart, and lungs. Only in the Thimerosal-treated animals were the lesions in the lungs numerous or severe enough to warrant comment (see Table 6). Here only disease incidence in the high dose of each compound is recorded. The three compounds chosen had the highest incidence of bronchopneumonia and in comparison with the controls it is evident that Thimerosal had a damaging effect on the lung or its defense apparatus. Since the death rate in this group paralleled the deaths in the other compounds, it must be concluded that the damage was slight, continuous, and perhaps cumulative. The incidence of pneumonia within the four dose levels of the Thimerosal group was dose-related.

Carcinogenicity

The outstanding result was the occurrence of 26 sarcomas at the injection site of benzethonium chloride out of 200 injected animals (13%). All the other test compounds had from two to four such tumors (1% to 2%). This is strongly correlated with the high incidence of granulomatous reactions to the subcutaneous injection of the compound. This response was dose-related, and at the highest dose the indurations persisted for 10 to 12 months. These sarcomas were principally fibrosarcomas showing very little tendency to metastasize but grew steadily to a larger size. The majority of these tumors developed during the last nine months of the trial.

The incidence of other tumors was carefully recorded. Each animal on trial was autopsied either at 12 months or at 18 months as planned. All spontaneous deaths, moribund animals, and those showing pathology or abnormal organ weights were histologically examined in addition to those chosen for routine examination. Every animal in the highest dose level was so processed. This led to the accumulation of a large number of incidental tumor findings.

Table 6
Incidence of Bronchopneumonia (18 months)^a

Treatment group	Total number of animals	No. of animals with bronchopneumonia		Percentage of animals with bronchopneumonia	
		Gross pathology	Histopathology	Gross pathology	Histopathology
Negative control	120	5	16	4%	13%
Vehicle control	120	3	9	3	8
Benzethonium					

compiled only for the highest dose levels of
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		Gross pathology	Histopathology	Gross pathology	Histopathology
Negative control	120	5	16	4%	13%
Vehicle control	120	3	9	3	8
Benzethonium chloride	80	2	3	3	4
Ethylene chlorohydrin	80	3	2	4	3
Thimerosal	80	39	48	49	60

^a High level of compounds compared with vehicle and negative controls.

Table 7
Tumor Incidence and Location

Treatment	% of tumor bearing rats ^a (all dose levels included)		Tumor location ^b (all dose levels included)					
			Male ^c		Female			
	Male	Female	Injection site	Other	Injection site	Mammary	Uterine	Other
Negative control	10	12	1/50	6/50	0/50	1/50	5/50	7/50
Vehicle control	6	14	0/50	3/50	0/50	3/50	5/50	8/50
Benzethonium chloride	19	18	16/100	6/100	10/100	5/100	4/100	3/100
Ethylene chlorohydrin	3	16	2/100	1/100	0/100	3/100	6/100	13/100
Ethylene glycol	5	12	2/100	4/100	0/100	5/100	11/100	6/100
Thiomersal	6	10	2/100	4/100	2/100	2/100	8/100	5/100
Methyl paraben	5	17	2/100	3/100	1/100	8/100	8/100	9/100
Phenol red	7	10	2/100	5/100	0/100	3/100	11/100	8/100
Pyridine	3	5	2/100	1/100	0/100	3/100	7/100	2/100

^a Excluding testicular interstitial cell tumors and uterine polyps.

^b Some rats had more than one tumor type.

^c Excluding testicular tumors.

Table 8
Tumor Incidence and Location

Compound	Pituitary (adenoma)			Adrenal			Blood (leukemia)		
	Male/Female	Tumor bearing	No. of rats	Male/Female	Tumor bearing	No. of rats	Male/Female	Tumor bearing	No. of rats
Negative control	0 1	1/120		1 0	1/120		0 2	2/120	
Vehicle control	1 1	2/100		1 0	1/100		0 1	1/100	
Benzethonium chloride	0 0	0/200		1 0	1/200		0 1	1/200	
Ethylene chlorohydrin	0 7	7/200		0 0	0/200		1 4	5/200	
Ethylene glycol	0 0	0/200		2 1	3/200		0 2	2/200	

Benzethonium chloride	19	18	16/100	00	10/100	5/100	4/100	3/100
Ethylene chlorohydrin	3	16	2/100	1/100	0/100	3/100	6/100	13/100
Ethylene glycol	5	12	2/100	4/100	0/100	5/100	11/100	6/100
Thiomerosal	6	10	2/100	4/100	2/100	2/100	8/100	5/100
Methyl paraben	5	17	2/100	3/100	1/100	8/100	8/100	9/100
Phenol red	7	10	2/100	5/100	0/100	3/100	11/100	8/100
Pyridine	3	5	2/100	1/100	0/100	3/100	7/100	2/100

^a Excluding testicular interstitial cell tumors and uterine polyps.

^b Some rats had more than one tumor type.

^c Excluding testicular tumors.

MASON, CATE, AND BAKER

Table 8
Tumor Incidence and Location

Compound	Pituitary (adenoma)			Adrenal			Blood (leukemia)		
	Male/Female	Tumor bearing/No. of rats		Male/Female	Tumor bearing/No. of rats		Male/Female	Tumor bearing/No. of rats	
Negative control	0 1	1/120		1 0	1/120		0 2	2/120	
Vehicle control	1 1	2/100		1 0	1/100		0 1	1/100	
Benzethonium chloride	0 0	0/200		1 0	1/200		0 1	1/200	
Ethylene chlorohydrin	0 7	7/200		0 0	0/200		1 4	5/200	
Ethylene glycol	0 0	0/200		2 1	3/200		0 2	2/200	
Thimerosal	0 0	0/200		0 1	1/200		0 1	1/200	
Methyl paraben	2 2	4/200		0 0	0/200		0 1	1/200	
Phenol red	0 3	3/200		1 0	1/200		1 2	3/200	
Pyridine	0 1	1/200		0 0	0/200		0 1	1/200	
Totals	3 15	18/1620		6 2	8/1620		2 15	17/1620	

TOXIC AND CARCINOGENIC CHEMICALS IN VACCINES

Table 9
Tumor Incidence

Group	Dose	Animal tested	Tumor bearing rats ^a						Tumor types ^b					
			Male		Female		Both		Fibroma or sarcoma		Mammary		Others	
			No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
Test compound: negative control														
A	0	50	5	10					1	2	0	0	4	8
B	0	50			9	18					1	2	8	16
Total							14	14	1	1	1	1	12	12
Test compound: vehicle control (saline)														
A	0.25	50	3	6					0	0	0	0	3	6
B	0.25	50			9	18			0	0	3	6	6	12
Total							12	12	0	0	3	3	9	9
Test compound: benzethonium chloride														
A	3.0	80	11	28	10	25	21	26	16	20	2	3	6	8
B	1.0	60	6	20	4	13	10	17	8	13	2	3	3	5
C	0.3	40	2	10	3	15	5	13	2	5	1	3	3	8
D	0.1	20	0	0	1	10	1	5	0	0	1	5	0	0
Test compound: ethylene chlorohydrin														
A	10.0	80	1	3	7	18	8	10	1	1	1	1	6	8
B	3.0	60	1	3	9	30	10	17	1	2	2	3	7	12
C	1.0	40	1	5	4	20	5	13	0	0	0	0	5	13
D	0.3	20	0	0	1	10	1	5	0	0	0	0	1	5
Test compound: ethylene glycol														
A	1000.0	80	2	5	6	15	8	10	2	3	2	3	8	10
B	300.0	60	1	3	9	30	10	17	1	2	2	3	7	12
C	100.0	40	2	10	3	15	5	13	0	0	1	3	4	10
D	30.0	20	0	0	1	10	1	5	0	0	1	5	1	5
Test compound: thimerosal														
A	1.0	80	4	10	7	18	11	14	4	5	0	0	11	14
B	0.3	60	2	7	6	20	8	13	1	2	3	5	6	10
C	0.1	40	0	0	3	15	3	8	0	0	0	0	3	8
D	0.03	20	0	0	0	0	0	0	0	0	0	0	0	0
Test compound: phenol red														
A	1.0	80	2	5	4	10	6	8	0	0	1	1	7	9
B	0.56	60	4	13	8	27	12	20	2	3	2	3	10	17
C	0.32	40	1	5	1	5	2	5	0	0	0	0	3	8
D	0.18	20	0	0	2	20	2	10	0	0	0	0	2	10

Test compound: vehicle control (saline)

A	0.25	50	3	6					0	0	0	0	3	6
B	0.25	50			9	18			0	0	3	6	6	12
Total							12	12	0	0	3	3	9	9

Test compound: benzethonium chloride

A	3.0	80	11	28	10	25	21	26	16	20	2	3	6	8
B	1.0	60	6	20	4	13	10	17	8	13	2	3	3	5
C	0.3	40	2	10	3	15 ^a	5	13	2	5	1	3	3	8
D	0.1	20	0	0	1	10	1	5	0	0	1	5	0	0

Test compound: ethylenè chlorohydrin

A	10.0	80	1	3	7	18	8	10	1	1	1	1	6	8
B	3.0	60	1	3	9	30	10	17	1	2	2	3	7	12
C	1.0	40	1	5	4	20	5	13	0	0	0	0	5	13
D	0.3	20	0	0	1	10	1	5	0	0	0	0	1	5

Test compound: ethylene glycol

A	1000.0	80	2	5	6	15	8	10	2	3	2	3	8	10
B	300.0	60	1	3	9	30	10	17	1	2	2	3	7	12
C	100.0	40	2	10	3	15	5	13	0	0	1	3	4	10
D	30.0	20	0	0	1	10	1	5	0	0	1	5	1	5

Test compound: thimerosal

A	1.0	80	4	10	7	18	11	14	4	5	0	0	11	14
B	0.3	60	2	7	6	20	8	13	1	2	3	5	6	10
C	0.1	40	0	0	3	15	3	8	0	0	0	0	3	8
D	0.03	20	0	0	0	0	0	0	0	0	0	0	0	0

Test compound: phenol red

A	1.0	80	2	5	4	10	6	8	0	0	1	1	7	9
B	0.56	60	4	13	8	27	12	20	2	3	2	3	10	17
C	0.32	40	1	5	1	5	2	5	0	0	0	0	3	8
D	0.18	20	0	0	2	20	2	10	0	0	0	0	2	10

Test compound: methyl paraben

A	3.5	80	3	8	6	15	9	11	1	1	4	5	5	6
B	2.0	60	1	3	10	33	11	18	2	3	3	5	8	13
C	1.1	40	1	5	2	10	3	8	0	0	0	0	3	8
D	0.6	20	0	0	5	50	5	25	0	0	1	5	4	20

Test compound: pyridine

A	100.0	80	2	5	2	5	4	5	2	3	0	0	2	3
B	30.0	60	1	3	7	23	8	13	0	0	2	3	6	10
C	10.0	40	0	0	2	10	2	5	0	0	0	0	2	5
D	3.0	20	0	0	1	10	1	5	0	0	1	5	0	0

^aExcluding testicular tumors.^bSome animals had more than one tumor type.

Mammary fibroadenomas were encountered within each group and the incidence usually varied between 2% and 5%. Only the methyl paraben group showed an incidence of 8%.

Uterine polyps were encountered in many rats and the incidence varied from 4% to 11% in the test group as compared with 10% in the controls.

Pituitary adenomas occurred in many groups but only ethylene chlorohydrin had seven in the 100 females with none occurring in the males. Of the 18 pituitary adenomas seen in this study 15 occurred in females.

Of the eight adrenal tumors seen, six occurred in males but no test group had an outstanding number.

Leukemias were discovered by damage to liver, and enlargement of the lymph glands and spleen. Seventeen such cases were found and 15 of these occurred in females.

The tables (Tables 7-9) showing the tumor types, location, size, and metastases are grouped for each of the test compounds.

Testicular Tumors

In a previous study at this laboratory, Fischer rats were used in a long-term carcinogen trial. At the end of the study, a great deal of information was gathered on the incidence of spontaneous interstitial cell tumors (I.C.T.) in this strain of rats. Some of the results were reported by Hadidian et al. [2], who found that "Both vehicle and untreated control male rats exhibited a progressively increasing proportion of interstitial cell tumors of the testis. This lesion was diagnosed in almost all male autopsied at 600 days."

Other studies in our laboratory have confirmed the fact that I.C.T. of the testis occur with high frequency in male Fischer rats older than 500 days. At 600 days more than 95% have some tumors and more than 85% have tumors bilaterally.

In the present study rats which received the first five compounds listed in Table 10 have an incidence of I.C.T. comparable to control animals and no single dosage group of animals had an incidence lower than 80%. Of these compounds none showed any tendency toward accelerating the growth or invasiveness of the testicular tumor.

However, benzethonium chloride significantly depressed the incidence of I.C.T. at the two highest levels used (3.0 and 1.0 mg/kg) ($p < 0.01$).

Furthermore, there was a dose-related inhibition of spontaneous I.C.T. in Thimerosal-injected animals. At the highest dose level only four out of 27 male rats showed any I.C.T. This is a decrease from 100% in control animals to 14.8% ($p < 0.01$). As the dose of Thimerosal decreases the incidence of I.C.T. rapidly increases. Even at the lowest dose there is still

Table 10
Testicular Tumors (I.C.T.)
(588-602 Days)

Treatment		No. c started
Group	Dose, mg/kg	
Untreated controls		47
Vehicle controls		46
Ethylene chlorohydrin		87
Ethylene glycol		86
Thimerosal		86
Phenol red		86
Pyridine		86
Benzethonium chloride		
A	3.0	24
B	1.0	27
C	0.3	19
D	0.1	10
Thimerosal		
A	1.0	27
B	0.3	28
C	0.1	19
D	0.03	11

a noteworthy inhibition in that many ca single testis rather than being bilateral.

Discussion

The significance of the relatively high repeated injections of benzethonium chl The correlation is great between a very sequent granulomas, and the gradual de is also no doubt that there is a dose rel: also noteworthy that in only one instan injection site tumors of metastasis eithe any internal organ.

This type of induced neoplasm has t Goldberg [3] and by Grice and Mannel cells in the area of repeated irritation.

By definition a compound is carcinc

encountered within each group and the 2% and 5%. Only the methyl paraben

ed in many rats and the incidence varied as compared with 10% in the controls. many groups but only ethylene chloro-les with none occurring in the males. Of this study 15 occurred in females. en, six occurred in males but no test

damage to liver, and enlargement of the seen such cases were found and 15 of these

g the tumor types, location, size, and of the test compounds.

oratory, Fischer rats were used in a long- of the study, a great deal of informat on spontaneous interstitial cell tumors (I.C.T.) e results were reported by Hadidian et al. le untreated control male rats exhibited ion. interstitial cell tumors of the testis. ost all male autopsied at 600 days." y have confirmed the fact that I.C.T. of ncy in male Fischer rats older than 500 5% have some tumors and more than 85%

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ide significantly depressed the incidence of sed (3.0 and 1.0 mg/kg) ($p < 0.01$). e-related inhibition of spontaneous I.C.T. in the highest dose level only four out of

This is a decrease from 100% in control s the dose of Thimerosal decreases the ases. Even at the lowest dose there is still

Table 10
Testicular Tumors (I.C.T.) in Fischer Rats
(588-602 Days Old)

Treatment		No. of rats		% of rats with I.C.T.
Group	Dose, mg/kg	started	with I.C.T.	
Untreated controls		47	47	100.0
Vehicle controls		46	45	97.8
Ethylene chlorohydrin		87	83	95.4
Ethylene glycol		86	82	95.3
Thimerosal		86	80	93.0
Phenol red		86	82	95.3
Pyridine		86	82	95.3
Benzethonium chloride				
A	3.0	24	19	79.1
B	1.0	27	21	77.1
C	0.3	19	17	89.4
D	0.1	10	10	100.0
Thimerosal				
A	1.0	27	4	14.8
B	0.3	28	16	57.1
C	0.1	19	16	84.2
D	0.03	11	8	81.8

83.7%

51.7%

a noteworthy inhibition in that many cases of I.C.T. are confined to a single testis rather than being bilateral.

Discussion

The significance of the relatively high incidence of fibrosarcomas after repeated injections of benzethonium chloride deserves special consideration. The correlation is great between a very high rate of initial irritation, subsequent granulomas, and the gradual development of massive tumors. There is also no doubt that there is a dose relationship to tumor incidence. *It is also noteworthy that in only one instance was there evidence in the 41 injection site tumors of metastasis either to regional lymph glands or to any internal organ.*

This type of induced neoplasm has been well described by Grasso and Goldberg [3] and by Grice and Mannell [4] as arising from mesenchymal cells in the area of repeated irritation.

By definition a compound is carcinogenic if it produces a neoplasm. The

degree of carcinogenicity is modified by numerous factors such as the susceptibility of the species or strain, the dose, route, frequency of dosing, and the concentration of the test compound as well as the profile of resident viruses in the host. The time required for tumor inception should also be considered.

Benzethonium chloride should be classed as a relatively weak carcinogen (D_2) according to the classification proposed by Grasso and Golberg [5].

In examining the tumor occurrence with repeated large doses of Thimerosal, only five mesenchymal tumors occurred in the 200 injected animals. The four injection site-related tumors occurred in the 80 animals given the highest tolerated dose. It was at this dose that the largest numbers of inflammatory reactions were noted early in the treatment.

Of these tumors, only one (A-56) was outstanding for its malignant cytology. The others had well differentiated cell types between which there were large bundles of collagenous fibers and a very low incidence of mitotic figures.

SUMMARY

The toxic and carcinogenic potential of seven preservatives and extracting agents used in biological products were studied. The compounds were benzethonium chloride, ethylene chlorohydrin, ethylene glycol, Thimerosal (merthiolate), methyl paraben, phenol red, and pyridine.

Fischer rats were used in both the toxicity as well as the carcinogenic trials. The following table gives the single dose LD_{50} , the repeated dose-maximum tolerated dose, and the selected maximum dose for the twice weekly injection schedule that continued for one year.

Summary Toxicology Table

Compound	LD_{50} , mg/kg	Maximum tolerated dose, mg/kg	Maximum dose for 100 injections
Benzethonium chloride	119.0	3.0	3.0
Ethylene chlorohydrin	71.6	< 30.0	10.0
Ethylene glycol	5300.0	< 1700.0	1000.0
Thimerosal (merthiolate)	98.0	< 5.0	1.0
Methyl paraben	> 500.0	—	3.5
Phenol red	> 600.0	—	1.0
Pyridine	866.0	< 180.0	100.0

Two hundred animals were assigned for the study. One hundred and twenty animals were assigned as vehicle controls, while 160 rats were used as sulfide as a positive control.

The toxicity of the compounds given over the 18-month period exceeded the estimates based on the preliminary studies. In one year, the mortality of the treated groups was that of the negative and vehicle controls (2%) for the 18-month period at which time the average weight gain was 6.5% while the average of the controls was 10.5%. The toxicity of benzethonium chloride and Thimerosal at the highest dose levels decreased weight gains as compared with the controls respectively. At the lower dose levels all the animals gained weight similar to the controls.

The only remarkable histopathology was pneumonia which developed in many of the treated animals. It was clearly a dose-related finding.

Benzethonium chloride was the outstanding compound with 26 injection site-related tumors in the 200 injected animals. The other test compounds had from two to four such tumors. The high incidence of injection site tumors was due to the incidence of induration and granulomas caused by the subcutaneously injected compounds of which none metastasized. Thimerosal had the highest incidence of indurations and was second highest with fibrosarcomas.

Complete autopsies were carried out on all the animals at the end of the trial. Many tumors were observed throughout the body. Mammary fibroadenomas were common in the females and varied from 2% to 5%. Only the methyl paraben had an incidence of 8%.

Testicular tumors were found in most of the males at the end of 18 months. These are interstitial cell tumors. It is noteworthy that Thimerosal caused a decrease in the number of tumors.

Uterine polyps were seen commonly in the females. They had an incidence range of 4% to 11% as compared with the controls.

Eighteen pituitary adenomas were found in the females. These were in females. Ethylene chlorohydrin had none in the males. Of the eight adenomas in the males but there was no significance in the incidence. Thirteen leukemias were found distributed throughout the body.

ed by numerous factors such as the in, the dose, route, frequency of dosing, compound as well as the profile of response required for tumor inception should

be classed as a relatively weak carcinogen proposed by Grasso and Golberg [5].

ence with repeated large doses of tumors occurred in the 200 injected ated tumors occurred in the 80 animals t was at this dose that the largest num- e noted early in the treatment.

5) was outstanding for its malignant erentiated cell types between which there bers and a very low incidence of

SUMMARY

ential of seven preservatives and extracting were studied. The compounds were nic hydrin, ethylene glycol, Thimerosal no. 1, and pyridine. he toxicity as well as the carcinogenic e single dose LD₅₀, the repeated dose-selected maximum dose for the twice tinued for one year.

Toxicology Table

	Maximum tolerated dose, mg/kg	Maximum dose for 100 injections
.0	3.0	3.0
.6	< 30.0	10.0
.0	< 1700.0	1000.0
.0	< 5.0	1.0
.0	—	3.5
.0	—	1.0
.0	< 180.0	100.0

Two hundred animals were assigned for the testing of each compound. One hundred and twenty animals were assigned for each group of negative and vehicle controls, while 160 rats were used for the injection of nickel sulfide as a positive control.

The toxicity of the compounds given over a period of one year did not exceed the estimates based on the preliminary toxicity trials. At the end of one year, the mortality of the treated groups (1.85%) did not exceed that of the negative and vehicle controls (2.0%). This largely held true for the 18-month period at which time the treated groups had a mortality of 6.5% while the average of the controls was 7.05%. After 18 months, benzethonium chloride and Thimerosal at their highest dose level showed decreased weight gains as compared with the controls of 12% and 22%, respectively. At the lower dose levels all the compounds showed weight gains similar to the controls.

The only remarkable histopathology was related to a late broncho-pneumonia which developed in many of the Thimerosal-treated animals. It was clearly a dose-related finding.

Benzethonium chloride was the outstanding compound which gave rise to 26 injection site-related tumors in the 200 treated animals. All the other test compounds had from two to four such tumors, while the controls had one. The high incidence of injection site tumors was correlated with a high incidence of induration and granulomas caused by the irritating activity of the subcutaneously injected compounds. The tumors were fibrosarcomas of which none metastasized. Thimerosal had numerous injection site indurations and was second highest with fibromas.

Complete autopsies were carried out on almost all of the 1800 rats in the trial. Many tumors were observed that had no relation to the injection site. Mammary fibroadenomas were common to all groups and the incidence varied from 2% to 5%. Only the methyl paraben group showed an incidence of 8%.

Testicular tumors were found in most of the males that lived to 18 months. These are interstitial cell tumors peculiar to the Fischer rat. It is noteworthy that Thimerosal caused a dose-related inhibition of these tumors.

Uterine polyps were seen commonly in all groups, and the test groups had an incidence range of 4% to 11% as compared to 10% in the controls.

Eighteen pituitary adenomas were found in the 1800 rats. Fifteen of these were in females. Ethylene chlorohydrin had 7 in the 100 females with none in the males. Of the eight adrenal tumors seen, six occurred in males but there was no significance in the test group incidence. Seventeen leukemias were found distributed throughout all the groups. The

only noteworthy finding here was that 15 out of the 17 occurred in females.

Nickel sulfide served well as a positive control since over 90% of the Fischer rats showed characteristic sarcoma formation at the site of injection [1]. The sarcomas were pleomorphic with variations from the rhabdomyosarcomas to the more collagenous fibrosarcomas. Many of these were metastatic, especially to the lungs.

REFERENCES

- [1] W. C. Hueper, "Experimental studies in metal carcinogenesis: 1. Nickel cancer in rats," *Texas Rept. Biol. Med.*, 10:167-186 (1952).
- [2] Z. Hadidian, et al., "Test for chemical carcinogens," *J. Natl. Cancer Inst.*, 41:985-1036 (1968).
- [3] P. Grasso and L. Golberg, "Early changes at the site of repeated subcutaneous injection of food colorings," *Food Cosmet. Toxicol.*, 4, 269-282 (1966).
- [4] H. C. Grice and W. A. Mannell, "Rhabdomyosarcomas induced in rats by intramuscular injections of Blue VRS," *J. Natl. Cancer Inst.*, 37, 845 (1966).
- [5] P. Grasso and L. Golberg, "Subcutaneous sarcoma as an index of carcinogenic potency," *Food Cosmet. Toxicol.*, 4, 297-320 (1966).

100-Day LD₅₀ Index of Chronic Toxicity*

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INTRODUCTION

The principals involved in uniposal prediction of acute single-dose toxicity are complex. If the discipline of factorial toxicometrics, and its reactions, is thoroughly understood, estimation of toxicity can be duplicated with reasonable accuracy. The discipline of toxicometrics, or methods of measuring toxicity, has advanced at a slower pace, particularly in work involving multiposal than in uniposal.

The basic question to be answered in multiposal studies is how much of an agent can be given without producing what toxic effects. Because of the high cost, selection of daily doses is extremely difficult. The method used to select the daily dose: (a) multiple of the therapeutic dose, (b) increasing fractions of the acute LD₅₀, (c) decreasing fractions of the acute LD₅₀. That method (c) yields the most information.

The next question to consider is the role of

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Toxicity of Quaternaries

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QUATERNARY ammonium bactericides are employed widely as disinfectants and sanitizers in fields related to public health. Their major applications are (1) in the restaurant field as terminal sanitizers on dishes, glassware and eating utensils, (2) in the dairy field primarily in sanitizers and detergent-sanitizers used on milking equipment, (3) as janitorial disinfectants and deodorants, (4) in food plants as terminal sanitizers on equipment, (5) in hospitals as general disinfectants, (6) in barber shops and beauty parlors for equipment disinfection, (7) for disinfection and sanitization of fabrics such as baby diapers, (8) for mold control in food storage rooms, and (9) for algae control in swimming pools. In addition, these products find volume usage in the poultry and veterinary field as chicken drinking water sanitizers, in egg handling, both on the farm and in egg breaking plants, as general farm disinfectants, and as topical antiseptics. The drug industry also makes use of these compounds as anti-bacterial agents in formulations employed as skin antiseptics, particularly for the control of diaper rash on babies.

In practically all of these applications, contact with the skin, either incidental or functionary, is encountered, and in many cases the possible accidental ingestion of relatively sizable quantities must be recognized. We must also be realistic in accepting the possibility of accidental contamination of foodstuffs due to improper rinsing of equipment following quaternary use.

Having before us the possible contamination of foodstuffs, accidental oral ingestion, and skin exposure, we must consider the implications from the standpoint of toxic effects. We should know the acute oral toxicity of these compounds, and whether skin irritation or sensitization is a factor. We should know whether skin absorption creates any hazards. Finally, we should have a clear idea of the chronic toxicity of these compounds, to determine whether any deleterious effects can be expected from their continuous ingestion in foods or water over a long period of time. The significance of such knowledge gained about these products is related to the actual amount of quaternary ammonium compound that we could expect to find on equipment following sanitization. This has been determined on glassware prewashed with detergent, rinsed with 200 ppm quaternary solution (the normal sanitizing concentration) and allowed to drain for

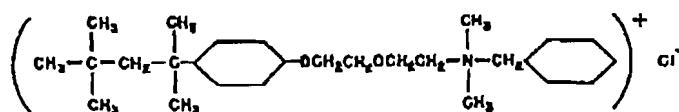
two minutes. Because the glassware was not drained dry, the values found were considered to be maximum. Utilizing a modification of the Harper, Elliker, and Mosely titrimetric procedure, concentrations ranging from only 0.24 ppm to 0.36 ppm quaternary were found in the solution when the glasses were filled with distilled water. Incidentally, both anionic and nonionic detergents were employed in prewashing the glasses; those washed with anionic gave generally slightly lower results.

The two well known commercial quaternary ammonium chlorides shown in figure 1, "Hyamine 1622" (diisobutyl phenoxy ethoxy ethyl dimethyl benzyl ammonium chloride), and "Hyamine 2389" (alkyl C₉-C₁₅ tolyl methyl trimethyl ammonium chlorides) manufactured by the Rohm and Haas Company, were submitted to a series of tests. "Hyamine 1622" is supplied as a crystalline material,

Figure 1

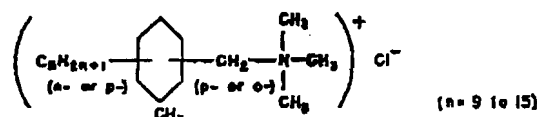
HYAMINE 1622

DI-ISOBUTYL PHENOXY ETHOXY ETHYL DIMETHYL BENZYL AMMONIUM CHLORIDE



HYAMINE 2389

ALKYL (C₉ to C₁₅) TOLYL METHYL TRIMETHYL AMMONIUM CHLORIDES



*Paper presented before the 40th Annual C.S.N.A. Meeting, Washington, Dec. 8, 1952.

Hyamine 1622 is a 30% concentrate in water solution.

of similar quaternary ammonium germicides in general have been quite limited in number (Alfredson et al., 1951; Fitzhugh and Nelson, 1948; Harshbarger, 1942; Shelanski, 1949; Woodward and Calvary, 1945) and "Hyamine 1622" and "Hyamine 2389" have been reported on only in a preliminary abstract (Finnegan et al., 1952).

Experimental Procedures and Results:

Acute Toxicity Experiments on Rats:

The acute toxicities of "Hyamine 1622" and of "Hyamine 2389" were determined on young adult male albino rats by the oral, intraperitoneal and intravenous routes. Ten rats were used at each of the significant points run on each sample. The LD₅₀'s as calculated by analysis of regression (Snedecor, 1946) using log-dose probit units are shown in Table I.

In the case of "Hyamine 2389," when given by the intravenous route, death resulted in a few minutes. With intraperitoneal

Table I

Acute Toxicity of Hyamine 1622 and Hyamine 2389 to Rats

Compound	Route of Administration	LD ₅₀ ± S.D. (mgm./kgm.)
1622	Oral	420 ± 25
	Intraperitoneal	33.1 ± 2.5
	Intravenous	19.1 ± 0.8
2389	Oral	389 ± 28
	Intraperitoneal	10.23 ± 1.00
	Intravenous	3.06 ± 0.13

administration most deaths occurred in 10 to 20 minutes. With oral administration time of death ranged from 10 to 60 minutes, only a few occurring later than this. In general, survival time was more prolonged with "Hyamine 1622." Although rats receiving the material intravenously usually died within 10 minutes a few of the deaths were delayed for several hours. By the intraperitoneal route death usually resulted within 24 hours. When administered orally a few deaths occurred within 24 hours, but about half of the deaths were delayed for one week. The maximum survival

time of those that ultimately died was 21 days. In all cases death was

was noted that the animals receiving "Hyamine 1622" intravenously quickly developed hematuria. Erythrocyte counts on some of these animals 48 hours after dosing showed values of approximately 6×10^6 , somewhat lower than normal but not severely so.

Chronic Toxicity Studies on Rats:

IDENTICALLY designed 2-year feeding experiments on rats were done on "Hyamine 1622" and "Hyamine 2389." For each compound a group of 60 male and 60 female albino rats of weaning age was divided into 12 colonies of 10 rats each (separated as to sex) and each rat individually caged. Finely ground "Purina Dog Chow Meal" served as a basic diet and into this was thoroughly mixed amounts of the "Hyamine" calculated to result in the following concentrations of the active ingredient: 0 (Control), 50, 200, 1000, 2500 and 5000 ppm (parts per million). One colony of each sex was placed on each dietary level.

Table II

Survival Data for Rats Receiving Hyamine 1622 or Hyamine 2389 in Their Diets for a Two-Year Period.

Compound	Sex	Dietary Concentration (p.p.m.)	Number of Survivors							
			1 wk.	5 wk.	10 wk.	30 wk.	50 wk.	70 wk.	93 wk.	104 wk.
Hyamine 1622	Male	0	10	10	10	10	9	9	7	5
		50	10	10	10	10	9	8	4	3
		200	10	10	10	10	10	7	6	3
		1000	10	10	10	10	10	8	8	5
		2500	10	10	10	10	10	10	8	6
		5000	10	10	10	5	5	5	3	3
	Female	0	10	10	10	10	10	9	8	4
		50	10	10	10	10	9	9	8	5
		200	10	9	9	9	9	9	8	4
		1000	10	10	10	9	9	8	6	3
		2500	10	10	10	10	10	10	8	8
		5000	10	9	9	5	4	2	2	2
Hyamine 2389	Male	0	10	10	10	9	9	8	4	2
		50	10	10	9	9	9	8	6	5
		200	10	10	10	10	10	8	6	3
		1000	10	10	10	10	10	9	9	5
		2500	10	10	10	10	9	9	8	7
		5000	7	2	2	0	0	0	0	0
	Female	0	10	10	10	10	8	7	5	2
		50	10	10	10	10	9	9	7	6
		200	10	10	10	10	10	8	7	7
		1000	10	10	10	10	10	10	10	7
		2500	10	9	9	9	9	9	7	4
		5000	7	2	2	0	0	0	0	0

Survival data for each colony at representative time intervals are shown in Table II. It would appear that mortality is not appreciably affected by either "Hyamine" until a dietary level of 2500 ppm is exceeded. In the case of "Hyamine 1622" this mortality trend first became apparent at between 10 and 30 weeks, whereas with "Hyamine 2389" it appeared within one week and was very definite at 5 weeks and thereafter.

Table III presents growth data for each colony at representative time intervals. With "Hyamine 1622" growth did not appear to be significantly inhibited ($P = < .05$) until a dietary level of 2500 ppm was exceeded, and this inhibition was apparent as early as the first week. With Hyamine 2389 growth was inhibited at a level below that which produced increased mortality in that 2500 ppm brought about a decreased rate of growth that was statistically significant after about 50 weeks.

Erythrocyte counts, hemoglobin determinations and differential white blood cell counts were done during the eleventh and twenty-third months of feeding. All values

appeared to be within normal ranges.

Animals dying during the experiment that were not obviously autolyzed and all survivors of the 2-year period were necropsied and the following organs preserved in 10 per cent formaldehyde for histopathologic examination: heart, liver, lungs, thyroid, stomach, small intestine, cecum, large intestine, spleen, pancreas, kidneys, adrenals and gonads. Microscopic examination was made, in the main, on tissues from animals that either survived the experiment or died shortly before it terminated.

"Hyamine 1622" did not appear to produce any unique microscopic changes in rats until a dietary concentration of 2500 ppm was reached. One of the 6 males examined at this level and 2 of 3 males receiving 500 ppm displayed testicular atrophy. Of the 3 non-malignant tumors (mammary fibroadenomas) that were seen, none were found in animals on the 2 higher feeding levels. This occurrence of 3 tumors in the 46 animals that survived at least 100 weeks (6.5 per cent incidence) is in our experience low for rats of this age. The only

malignancy seen, a subcutaneous reticulum cell sarcoma found in one male receiving 200 ppm for 53 weeks, would appear to have no relationship to the Hyamine "1622" administration since no other such tumor was seen in animals receiving higher dietary levels for longer periods of time.

"Hyamine 2389" gave no indication of producing histopathologic changes at any of the levels fed. Five tumors (mammary fibromas and fibroadenomas) were found in the 31 rats studied that had survived at least 97 weeks, an incidence of 16 per cent. This is not an abnormally high incidence in our experience for rats of this age.

It was noted at the time of the first necropsies that animals on the higher feeding levels of either compound had ceca greatly distended by gas and very fluid contents. A similar finding has been described by Fitzhugh and Nelson (1943) and by Alfredson, et al., (1951) in rats fed alkylidimethylbenzylammonium chlorides. This condition was first seen in our studies at a level of 1000 ppm of each compound and became progressively more pronounced with increasing

Table III
Average Body Weight Data for Rats Receiving Hyamine 1622
or Hyamine 2389 in Their Diets for a Two-Year Period.

Compound	Sex	Dietary Concentration (p.p.m.)	Average Body Weight (gm.)								
			Start	1 wk.	5 wk.	10 wk.	30 wk.	50 wk.	70 wk.	90 wk.	104 wk.
Hyamine 1622	Male	0	48	74	224	333	494	522	545	547	535
		50	48	77	212	324	486	516	545	496	483
		200	48	76	216	322	468	517	560	579	576
		1000	48	79	216	332	516	566	637	593	577
		2500	48	74	211	330	456	506	552	542	478
		5000	48	61	167	278	353	389	373	384	401
	Female	0	46	72	156	201	269	300	314	345	283
		50	46	76	167	214	284	317	361	380	424
		200	46	69	154	202	260	294	326	343	339
		1000	46	73	158	208	282	324	370	358	375
		2500	46	68	145	188	246	284	328	342	347
		5000	46	56	137	186	221	229	232	252	257
Hyamine 2389	Male	0	67	101	224	313	474	502	522	520	438
		50	67	96	235	328	474	506	541	551	477
		200	67	101	233	337	494	535	589	504	507
		1000	67	95	219	321	478	521	583	552	520
		2500	68	88	193	303	419	460	473	503	471
		5000	67	59	80	168	—	—	—	—	—
	Female	0	60	90	157	199	260	293	325	351	350
		50	60	90	165	212	262	285	319	327	304
		200	61	84	149	197	257	289	340	344	362
		1000	61	87	153	194	267	299	356	399	401
		2500	60	79	146	191	244	263	272	306	311
		5000	60	59	127	175	—	—	—	—	

dietary concentration. Time of onset of enlargement was less than

regime, feces were expressed from each rat, placed in tared vials,

amination showed thinning of the cecal wall, no abnormal cytologic picture was seen. Figure 2 illustrates this condition in a male rat which was sacrificed after having been fed the 5000 ppm diet of Hyamine 1622 for 66 weeks.

Chronic Toxicity Studies on Dogs:

BOTH compounds were fed to dogs for 1 year at dietary levels of 5, 100, and 500 ppm. The diets were prepared as described above for the rat studies. Three adult mongrel dogs were placed on each concentration of each compound.

There were no deaths and all animals appeared well and gained weight during the experiment.

Prior to the start of the experiment and during the sixth and twelfth months, hemoglobin determinations and complete blood counts were done. All values appeared to be within normal limits.

At the end of the 1-year feeding period the dogs were necropsied and representative tissues (as listed for the rats) taken for histopathologic examination. No gross or microscopic abnormalities were observed in any of the dogs.

Studies on the Water Content of the Feces and Cecal Contents in Rats:

THESE studies were undertaken as a result of the observation that in the chronic rat feeding experiments the ceca were grossly distended at the higher feeding levels.

Sixty-six young male albino rats were individually caged and placed on a diet of finely ground Purina Dog Chow Meal for 10 days, and were divided into groups of 6. One group was continued on the control diet, and the other 10 groups were placed on diets containing 50, 200, 1000, 2500 and 5000 ppm. of "Hyamine 1622" or "Hyamine 2389." On the day before the rats were placed on the "Hyamine" diets and on the fourth, seventh and twelfth days of this

for 24 hours and reweighed to obtain moisture/dry weight ratios. In this study use was also made of the rats on all feeding levels of the 2-year program which at that time had received the experimental diets for 16 weeks. In addition, the moisture content of the cecal contents was determined in all of the animals on the 12 day experiment with the exception of those on the 5000 ppm. level of "Hyamine 2389" which had all died before that time. At the lower dietary levels (0, 50, 200 and 1000 ppm.) the total weight of the cecum and its contents was also determined.

Fecal moisture ratios in the 12 day experiment were significantly increased ($P < .05$) only at the two higher feeding levels (2500 and 5000 ppm) of both compounds, and this increase had reached its maximum by the time the first samples were taken after 4 days on the "Hyamine" diets. In the rats that had been on the 2-year feeding program for 16 weeks only those on the 5000 ppm levels of the "Hyamines" showed a significant increase in fecal moisture. Thus some adaptation appears to occur with continued exposure.

Significant increases in cecal moisture appeared in the rats receiving 2500 ppm of "Hyamine 1622" or "Hyamine 2389" for 12 days. This was also true at the 5000 ppm level of "Hyamine 1622." The increase in cecal moisture in the rats receiving 1000 ppm of the compounds was of borderline significance; below 100 ppm there was no significant change.

The total weights of ceca and contents increased significantly in the rats receiving 1000 ppm of the "Hyamines" for a period of 12 days but not for those receiving lower concentrations. Similar determinations were not made on rats receiving 2500 and 5000 ppm diets since visual inspection indicated that progressively greater increases occur at these levels.



Figure 2

Effect on Intestinal Flora Content in Rats:

THE known germicidal action of "Hyamine 1622" and "Hyamine 2389" raised the possibility that alteration in intestinal flora might be involved in the intestinal tract changes just described.

The same animals were used in this study that were used in the investigation on fecal and cecal water content. Immediately before placing the 12-day animals on the "Hyamine" diets and on the third, sixth and twelfth days of the experiment, freshly expressed feces were obtained from each rat for bacteriologic examinations. At the termination of the experiment samples of cecal contents were also taken. Bacteriologic examinations were also made on fresh feces from the rats that had been on the 0, 200 and 5000 ppm levels of the Hyamines for 16 weeks in the 2-year feeding program.

The feces or cecal contents were pooled as to dietary level, ground and placed in peptone broth. Ten successive 10-fold dilutions in broth were made and 1 ml. samples of each dilution were transferred to Petri dishes for preparation of pour plates. Two series of plates were made from each dilution. For aerobic bacterial counts the medium was dextrose-tryptose blood agar

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were prepared using anaerobic agar with dextrose (B.I.L.) and Brewer anaerobic Petri dish covers. All plates were incubated 48 hours at 37°C. The sum of the aerobic and anaerobic counts was considered as the total number of bacteria present.

The prevalence of the different types of aerobic bacteria was determined by preparing streak plates from the 1:10 and 1:100 dilutions on blood agar and eosin methylene blue agar plates. Identification was made by colony appearance with occasional preparation of Gram-stained smears of individual colonies for confirmatory purposes. In addition, the incidence of Gram positive to Gram negative bacteria was determined by examination of Gram-stained smears prepared from "standard" loopfuls of the 1:10 dilutions of fecal or cecal material spread evenly over the measured surface of glass slides. The variations in the protozoan population of fecal and cecal material was determined by examination of wet mounts from the 1:10 dilution.

The results of these experiments indicate that only the 2500 and 5000 ppm levels of either of the "Hyamines" produced a marked reduction in the Gram positive bacterial flora of the feces and cecal contents. At these same levels there also occurred a relative increase in the *E. coli* and/or *A. aerogenes* and *P. vulgaris* flora. It is interesting to note that these levels were those associated with changes in moisture of feces and cecal contents. The relative number of aerobic and anaerobic and the total number of bacteria was not altered nor were the protozoan fauna. Blood obtained by cardiac puncture from moribund animals of the 5000 ppm level was bacteriologically negative.

Effect on Tone and Motility of the Intestine:

It also seemed possible that the effect on the gut might be due to a direct decrease in tone or motility. Therefore, the effects of "Hyamine 1622" and "Hyamine 2389" on isolated

ISOLATED RABBIT INTESTINE; HYAMINE 1622

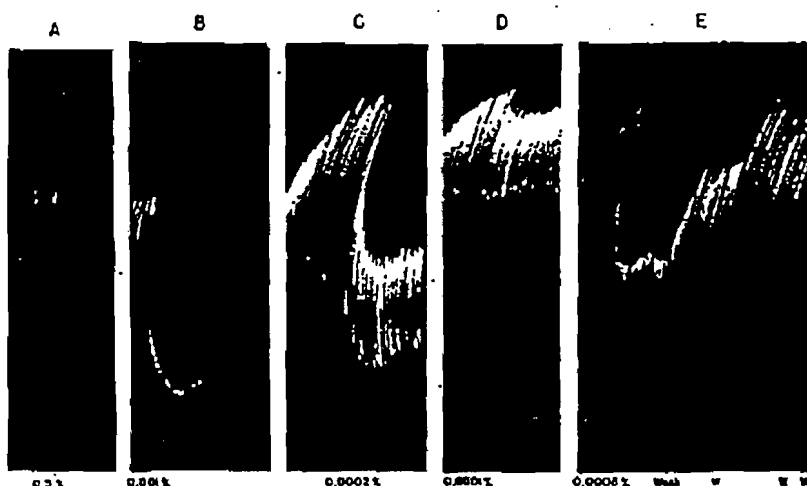


Figure 3

segments of rabbit and rat ileum were studied by the Magnus technic. Amounts of either "Hyamine 1622" or "Hyamine 2389" were added to the muscle bath so as to result in concentrations of from 0.5 per cent (5000 ppm) down to 0.00005 per cent (0.5 ppm). Fresh segments were used for each concentration.

Both compounds appeared to be of equal potency in their inhibition of the smooth muscle of the rabbit ileum. Concentrations of either agent down through 0.002 per cent produced cessation of contractions and greatly decreased the tone of the muscle. At 0.001 per cent tone was equally depressed but a slight degree of motility remained. At 0.0002 per cent, although tone was still quite markedly depressed, motility was not severely diminished. At 0.0001 per cent there was but slight effect on muscle tone and no effect upon the rhythmic movements. The 0.00005 per cent concentration caused no effect on either activity. An example of these effects is shown in Figure 3. Rat ileum reacted in a similar fashion but seemed slightly more sensitive to these agents.

The "Hyamines" did not act as general protoplasmic poisons on intestinal smooth muscle but rather appeared to have a more specific

effect. The depression produced by concentrations as high as 0.005 per cent in the muscle bath could be reversed by 2 or 3 changes of Locke-Ringer's solution, the muscle being restored to its original state of tone and motility (see part E, Figure 3).

Ganglionic Blocking Action:

THE quaternary ammonium structure of the "Hyamines" suggested that they may have some degree of autonomic blocking action. Investigation of this possibility was carried out with both compounds in dogs. The animals, anesthetized with Dial-urethane, were arranged for recording carotid arterial pressure. The pressor response to a fixed intravenous dose of epinephrine and of nicotine was first determined. The "Hyamine" was then administered intravenously, and following restabilization of blood pressure the previously used doses of epinephrine and nicotine were given. Since nicotine produces its pressor action through ganglionic stimulation

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Marketing . . .

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cases out of ten the poor results obtained were caused by not reading and following label directions. It's

Quaternaries

tion, abolition of the pressor response to nicotine with retention of the direct cardio-vascular effect of epinephrine would indicate ganglionic blockade. Using this method it was found that both "Hyamines" almost completely blocked sympathetic ganglia in a dose of 2 mgm./kgm. An example of this effect is shown in Figure 4. In the case of "Hyamine 1622" this effect persisted for about 5 hours in 2 of the 4 dogs to which it was administered. The duration of blockade with "Hyamine 2389" was 2-3 hours. In a dose of 1 mgm./kgm. of either agent, blockade is less complete and of shorter duration. Intravenous administration of either "Hyamine" in the 2 mgm./kgm. dosage is accompanied by a sharp but transient fall in blood pressure; with larger doses (4 mgm./kgm.) death ensues.

Irritant Effects on the Rabbit Eye:

THE irritating potency of various concentrations of both Hyamines was studied by instilling several drops in the rabbit eye and observing the conjunctival mucosa at intervals thereafter. The object of the experiment was to determine the concentration which would just produce perceptible irritation in the form of erythema and below which this did not occur. Five animals were used at each concentration. With Hyamine 1622 this concentration lay between 0.01 and 0.03 per cent, and with "Hyamine 2389" between 0.03 and 0.1 per cent.

Local and Systemic Effects of Daily Application to the Skin of the Rabbit:

SIX albino rabbits were used for each compound. The hair was closely clipped from the back and sides from the base of the neck to the hind legs. To one group 2 ml. of 0.1 per cent "Hyamine 1622" was applied to the clipped area once daily, 5 days a week for 4 weeks.

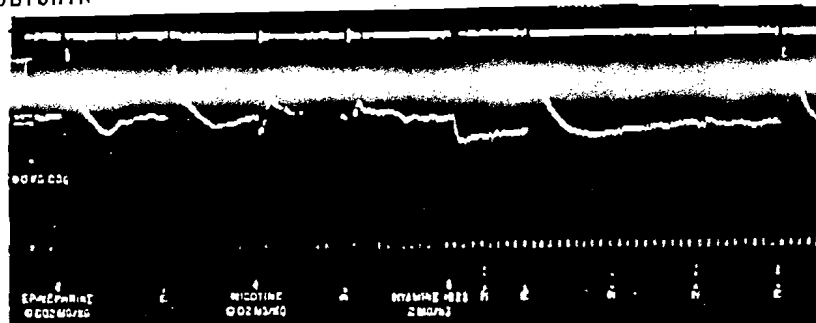


Figure 4

The other group received 0.05 per cent "Hyamine 2389" on the same schedule. No signs of skin irritation or of systemic effects were observed. At the end of the treatment period the animals were sacrificed and skin and other tissues (as listed above for rats) submitted to histopathologic examination. No gross or microscopic abnormalities were found.

Skin Irritation and Sensitization Produced by Hyamine 2389 in Man:

TWO separate patch test experiments were done on 50 human subjects each to determine the irritant properties of "Hyamine 2389." In all cases the material was applied by saturating a one-quarter inch square of cotton cloth with the solution and placing this on the volar surface of the forearm. This, in turn, was covered with a 1 inch square of cellophane that was held in place with a 2 inch square of adhesive tape. The patches were left in place for 48 hours, and on removal the area under each patch was examined for signs of irritation.

The first of these experiments was designed to investigate both primary irritation and the possibility of sensitization. Forty-two subjects received 50 per cent solutions and 8 received 10 per cent solutions. In the higher concentration 10 individuals showed positive reactions, which in most cases took the form of mild erythema. However, in 1 instance such severe erythema and weeping edema was seen as to discourage the further use of this individual as a subject. In the

subjects receiving 10 per cent solutions, 2 positive reactions were seen and both of these were of a mild nature.

Two weeks later 49 of the subjects were repatched on the other arm with a 10 per cent solution of "Hyamine 2389," the purpose being to determine the possibility of sensitization to the compound. In those individuals who had originally received the 50 per cent solutions there were 13 positive reactions and none of these was a severe response. In those originally receiving 10 per cent, the second application produced one mild positive reaction. It was concluded that these reactions represented primary irritation and not sensitization.

A second experiment was done to determine whether or not lower concentrations of "Hyamine 2389" would produce primary irritation. A second patch for testing sensitization was not applied. To the forearms of 50 additional subjects 4 patches were applied. The cotton squares were saturated with 3, 1 and 0.02 per cent solutions of Hyamine 2389; water was used on the fourth patch as a control. When the patches were removed 48 hours later no evidence of irritation could be seen in any of the areas.

Discussion:

THE data presented define rather clearly the nature of the toxicological characteristics of these two quaternary ammonium bactericides. Some conclusions can be drawn, based on these characteristics, as to the safety of these products for the various uses detailed earlier in the report.

1. The use of concentrations of 200 ppm employed in sanitization of restaurant utensils and dishes, food handling equipment, etc., represents no hazard from an acute toxicity standpoint, from the standpoint of irritation to the hands, and from the standpoint of skin absorption. This is important in that dishwashers and similar workers have occasion to keep their hands immersed in the sanitizing solution over long periods of time and at frequent intervals.

2. Concentrated material, although representing no major hazard, does require the normal care in manufacture in the plant, and it is indicated that skin contact should be avoided, and steps should be taken to prevent splashing into the eye. When the material is brought into contact with either the skin or the eye, the affected parts should be washed immediately.

3. The introduction of quaternary ammonium germicides, either willfully or accidentally to food products is unlawful but it is consequential and comforting to know from the chronic toxicity data that there is no hazard involved should such adulteration take place. It was indicated earlier in this report that less than 1 ppm of quaternary could

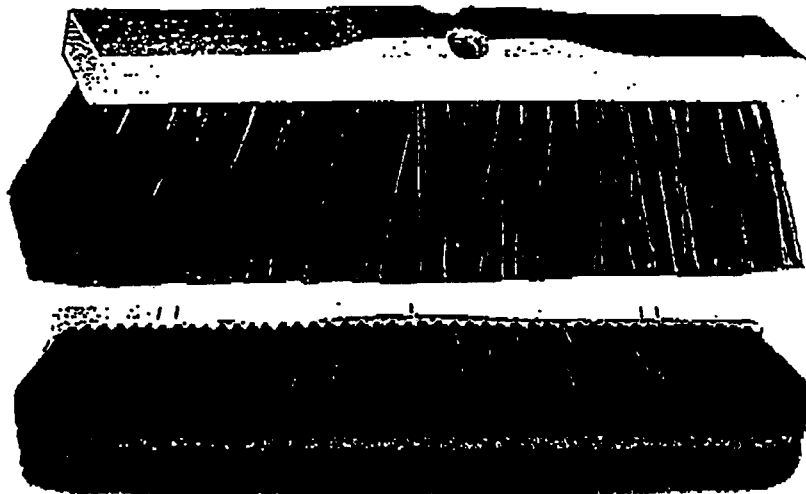
be recovered from glass tumbler sanitized with a 200 ppm solution of quaternary. It is doubtful that in normal plant practice more than this amount could be picked up from plant equipment which had not been properly rinsed with fresh water. The willful addition of quaternary to food in substantial amounts would be readily detected because of taste problems. Various studies have been made by workers in this field and it is the general consensus that the bitter and astringent taste of quaternaries is readily discernible in food products when as little as 10 to 20 ppm are added.

References

- Alfredson, B. V., Siefel, J. R., Thorp, E. Jr., Baten, W. D. and Gray, M. L.: *J. Am. Pharm. A. (Scient. Ed.)*, 40:263, 1951.
- Finnegan, J. K., Larson, P. S., Smith, R. G., Jr., Haug, H. B., Reil, J. D. and Dreyfuss, M. L.: *Fed. Proc.*, 11:345, 1952.
- Fitzhugh, O. G. and Nelson, A. A.: *J. Am. Pharm. A. (Scient. Ed.)*, 37:29, 1948.
- Harshbarger, K. E.: *J. Dairy Sci.*, 25:169, 1942.
- Shelanski, H. A.: *Soap Sanit. Chem.*, 25:125, 1949.
- Snedecor, G. W.: "Statistical Methods," Iowa State College Press, Ames, 4th Ed., 1946.
- Woodard, G. and Calvery, H. O.: *Proc. Scient. Sec. Toilet Goods Assoc.*, No. 3, 1945.

Two new brushes introduced recently by Moran Brush Manufacturing Co., Hamden, Conn., are: top, the "Atlas Garage Brush," and, below, "Wyette Floor Brush." The garage brush is made of heavy gauge "Algil" plastic, which is not affected by gasoline, kerosene or oil. It may be used on wet or dry surfaces. It also doubles as a snow brush. The garage brush is available in sizes from 14 to 36 inches.

The "Wyette" is a brush designed especially for factory use. It is constructed with a middle row of bright tempered steel wire for removing heavily caked dirt. Four surrounding rows of stiff black Tampico take care of medium dirt and a casing of horse hair and "Saran" mixture removes fine dust. This brush may be used on any concrete, cement, brick or uneven wood floor.



Guy Robbins Dies

Guy P. Robbins, 69, president of George B. Robbins Disinfectant Co., Cambridge, Mass., died after a lingering illness, Dec. 16. He is a past eastern regional vice-president of the National Sanitary Supply Association.

In addition to his widow, Donna, Mr. Robbins is survived by his son, Paul, who is connected with George B. Robbins Co., and a daughter.

Diatomite . . .

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removal of surface finish and also because of its good suspension qualities due to extreme fineness and relatively light density. For a badly chalked surface, abrasive action may not be as rapid as desired and a coarser and harder grade of diatomite can be used for more rapid results.

Modern cleaner and polish formulations are designed either to maintain the diatomite permanently in suspension in a semi-gel-like liquid or if permitting the diatomite to settle out wholly or partially on standing, cause it to form a soft flocculated settled portion which is resuspended readily on shaking. The amount of diatomite used is in the range of 10 to 20 per cent, usually about 15 per cent. Composition of the liquid and amount and type of cleaning and dispersing agents also are very important in their effect on efficiency and satisfactory application qualities.

In the past 30-year period, during which diatomite automobile polish abrasives have been widely used, important improvements in the diatomite products have been made for better performance just as improvements have been made in this same period in the formulation of automobile cleaners and polishes. The efficient products available today make the task of automobile finish maintenance easier and more satisfactory than in the past as well as prolonging the effective life and better preserving the good appearance of modern finishes.

APPENDIX E

GENETIC TOXICOLOGY

<i>SALMONELLA</i> MUTAGENICITY TEST PROTOCOL	E-2
CHINESE HAMSTER OVARY CELL CYTOGENETICS PROTOCOLS	E-2
RESULTS	E-3
TABLE E1 Mutagenicity of Benzethonium Chloride in <i>Salmonella typhimurium</i>	E-4
TABLE E2 Induction of Sister Chromatid Exchanges in Chinese Hamster Ovary Cells by Benzethonium Chloride	E-6
TABLE E3 Induction of Chromosomal Aberrations in Chinese Hamster Ovary Cells by Benzethonium Chloride	E-7

GENETIC TOXICOLOGY

SALMONELLA MUTAGENICITY TEST PROTOCOL

Testing was performed as reported by Zeiger *et al.* (1987). Benzethonium chloride was sent to the laboratory as a coded aliquot from Radian Corporation (Austin, TX). It was incubated with the *Salmonella typhimurium* tester strains (TA98, TA100, TA1535, and TA1537) either in buffer or S9 mix (metabolic activation enzymes and cofactors from Aroclor 1254-induced male Sprague-Dawley rat or Syrian hamster liver) for 20 minutes at 37° C. Top agar supplemented with *l*-histidine and *d*-biotin was added, and the contents of the tubes were mixed and poured onto the surfaces of minimal glucose agar plates. Histidine-independent mutant colonies arising on these plates were counted following incubation for 2 days at 37° C.

Each trial consisted of triplicate plates of concurrent positive and negative controls and at least five doses of benzethonium chloride. The high dose was limited by toxicity. All positive trials were repeated under the conditions that elicited the positive response. If no positive responses were seen, all negative trials were repeated.

In this assay, a positive response is defined as a reproducible, dose-related increase in histidine-independent (revertant) colonies in any one strain/activation combination. An equivocal response is defined as an increase in revertants that is not dose related, not reproducible, or is of insufficient magnitude to support a determination of mutagenicity. A negative response is obtained when no increase in revertant colonies is observed following chemical treatment. There was no minimum percentage or fold increase required for a chemical to be judged positive or weakly positive.

CHINESE HAMSTER OVARY CELL CYTOGENETICS PROTOCOLS

Testing was performed as reported by Galloway *et al.* (1987). Benzethonium chloride was sent to the laboratory as a coded aliquot by Radian Corporation. It was tested in cultured Chinese hamster ovary (CHO) cells for induction of sister chromatid exchanges (SCEs) and chromosomal aberrations (Abs), both in the presence and absence of Aroclor 1254-induced male Sprague-Dawley rat liver S9 and cofactor mix. Cultures were handled under gold lights to prevent photolysis of bromodeoxyuridine-substituted DNA. Each test consisted of concurrent solvent and positive controls and of at least three doses of benzethonium chloride; the high dose was limited by toxicity. A single flask per dose was used.

Sister Chromatid Exchange Test: In the SCE test without S9, CHO cells were incubated for 26 hours with benzethonium chloride in McCoy's 5A medium supplemented with fetal bovine serum, *l*-glutamine, and antibiotics. Bromodeoxyuridine (BrdU) was added 2 hours after culture initiation. After 26 hours, the medium containing benzethonium chloride was removed and replaced with fresh medium plus BrdU and Colcemid, and incubation was continued for 2 hours. Cells were then harvested by mitotic shake-off, fixed, and stained with Hoechst 33258 and Giemsa. In the SCE test with S9, cells were incubated with benzethonium chloride, serum-free medium, and S9 for 2 hours. The medium was then removed and replaced with medium containing serum and BrdU and no benzethonium chloride and incubation proceeded for an additional 26 hours, with Colcemid present for the final 2 hours. Harvesting and staining were the same as for cells treated without S9. All slides were scored blind and those from a single test were read by the same person. Fifty second-division metaphase cells were scored for frequency of SCEs/cell from each dose level.

Statistical analyses were conducted on the slopes of the dose-response curves and the individual dose points (Galloway *et al.*, 1987). An SCE frequency 20% above the concurrent solvent control value was chosen as a statistically conservative positive response. The probability of this level of difference occurring by chance at one dose point is less than 0.01; the probability for such a chance occurrence at two dose points is less than 0.001. An increase of 20% or greater at any single dose was considered weak evidence of activity; increases at two or more doses resulted in a determination that the trial was positive. A statistically significant trend ($P < 0.005$) in the absence of any responses reaching 20% above background led to a call of equivocal.

Chromosomal Aberrations Test: In the Abs test without S9, cells were incubated in McCoy's 5A medium with benzethonium chloride for 12 hours; Colcemid was added and incubation continued for 2 hours. The cells were then harvested by mitotic shake-off, fixed, and stained with Giemsa. For the Abs test with S9, cells were treated with benzethonium chloride and S9 for 2 hours, after which the treatment medium was removed and the cells were incubated for 12 hours in fresh medium, with Colcemid present for the final 2 hours. Cells were harvested in the same manner as for the treatment without S9.

Cells were selected for scoring on the basis of good morphology and completeness of karyotype (21 ± 2 chromosomes). All slides were scored blind and those from a single test were read by the same person. One hundred first-division metaphase cells were scored at each dose level. Classes of aberrations included simple (breaks and terminal deletions), complex (rearrangements and translocations), and other (pulverized cells, despiralized chromosomes, and cells containing 10 or more aberrations).

Chromosomal aberration data are presented as percentage of cells with aberrations. To arrive at a statistical call for a trial, analyses were conducted on both the dose response curve and individual dose points. For a single trial, a statistically significant ($P \leq 0.05$) difference for one dose point and a significant trend ($P \leq 0.015$) were considered weak evidence for a positive response; significant differences for two or more doses indicated the trial was positive. A positive trend test in the absence of a statistically significant increase at any one dose resulted in an equivocal call (Galloway *et al.*, 1987). Ultimately, the trial calls were based on a consideration of the statistical analyses as well as the biological information available to the reviewers.

RESULTS

Benzethonium chloride (0.010 to 100 $\mu\text{g}/\text{plate}$) was not mutagenic in *Salmonella typhimurium* strains TA98, TA100, TA1535, or TA1537 when tested with a preincubation protocol, with or without Aroclor 1254-induced male Sprague-Dawley rat or Syrian hamster liver S9 (Zeiger *et al.*, 1987; Table E1). In cytogenetic tests with cultured Chinese hamster ovary cells, benzethonium chloride did not induce SCEs (Table E2) or Abs (Table E3), with or without S9. Although an increase in chromosomal aberrations was observed in each of the two trials conducted, these increases were not statistically significant or dose related. No cell cycle delay was noted in either the Abs test or the SCE test.

TABLE E1
Mutagenicity of Benzethonium Chloride in *Salmonella typhimurium*^a

Strain	Dose (μ g/plate)	Revertants/plate ^b					
		-S9		+10% hamster S9		+10% rat S9	
		Trial 1	Trial 2	Trial 1	Trial 2	Trial 1	Trial 2
TA98	0.00	20 \pm 1.8	14 \pm 4.1	19 \pm 2.5	28 \pm 3.2	22 \pm 3.0	21 \pm 0.9
	0.01	21 \pm 0.7	7 \pm 1.2				
	0.03	18 \pm 1.8	6 \pm 0.7				
	0.10	19 \pm 2.1	7 \pm 0.3				
	0.30	18 \pm 0.9	10 \pm 1.8				
	1.00	22 \pm 1.5	10 \pm 4.2	15 \pm 1.9	28 \pm 4.1	21 \pm 2.0	17 \pm 1.9
	3.30			16 \pm 2.9	20 \pm 0.7	24 \pm 3.2	20 \pm 2.4
	10.00			16 \pm 1.7	20 \pm 1.3	24 \pm 1.5	16 \pm 1.8
	33.00			10 \pm 2.1	19 \pm 2.9	16 \pm 0.7	24 \pm 1.2
	100.00			7 \pm 1.2	21 \pm 0.9	18 \pm 0.7	18 \pm 3.5
Trial summary		Negative	Negative	Negative	Negative	Negative	Negative
Positive control		180 \pm 63.8	123 \pm 9.0	1,152 \pm 40.1	719 \pm 45.8	872 \pm 70.4	1,150 \pm 21.3
TA100	0.00	90 \pm 1.8	101 \pm 9.0	109 \pm 7.1	152 \pm 5.2	128 \pm 9.3	142 \pm 4.7
	0.01	87 \pm 0.3	92 \pm 3.8				
	0.03	85 \pm 4.2	107 \pm 0.3				
	0.10	82 \pm 5.2	103 \pm 5.3				
	0.30	94 \pm 4.7	99 \pm 4.3				
	1.00	92 \pm 5.0	66 \pm 1.7	105 \pm 1.5	130 \pm 5.9	109 \pm 7.4	122 \pm 8.0
	3.30			100 \pm 3.2	133 \pm 16.3	121 \pm 6.1	132 \pm 10.1
	10.00			94 \pm 2.3	140 \pm 14.2	118 \pm 13.0	144 \pm 6.9
	33.00			84 \pm 4.1	144 \pm 8.8	123 \pm 8.7	141 \pm 1.3
	100.00			34 \pm 3.8	143 \pm 15.2	128 \pm 6.0	toxic
Trial summary		Negative	Negative	Negative	Negative	Negative	Negative
Positive control ^c		936 \pm 11.1	1,024 \pm 18.1	1,636 \pm 125.5	1,021 \pm 63.2	1,264 \pm 205.4	2,105 \pm 62.1
TA1535	0.00	18 \pm 1.2	4 \pm 1.5	17 \pm 1.5	6 \pm 0.0	25 \pm 1.5	7 \pm 1.7
	0.01	14 \pm 1.9	4 \pm 0.7				
	0.03	16 \pm 3.7	4 \pm 1.2				
	0.10	14 \pm 3.2	5 \pm 0.7				
	0.30	8 \pm 1.5	2 \pm 1.2				
	1.00	8 \pm 0.6	2 \pm 0.6	18 \pm 3.3	4 \pm 1.8	16 \pm 0.9	5 \pm 1.9
	3.30			26 \pm 1.3	5 \pm 1.5	20 \pm 2.0	6 \pm 2.5
	10.00			15 \pm 4.2	3 \pm 0.9	17 \pm 1.5	5 \pm 1.7
	33.00			9 \pm 0.9	4 \pm 0.7	8 \pm 1.5	4 \pm 0.7
	100.00			toxic	toxic	toxic	toxic
Trial summary		Negative	Negative	Negative	Negative	Negative	Negative
Positive control		942 \pm 36.6	446 \pm 18.0	125 \pm 10.7	97 \pm 15.5	184 \pm 15.0	60 \pm 8.4

TABLE E1
Mutagenicity of Benzethonium Chloride in *Salmonella typhimurium* (continued)

Strain	Dose ($\mu\text{g}/\text{plate}$)	Revertants/plate					
		-S9		+10% hamster S9		+10% rat S9	
		Trial 1	Trial 2	Trial 1	Trial 2	Trial 1	Trial 2
TA1537	0.00	12 \pm 1.5	10 \pm 1.2	24 \pm 2.4	15 \pm 1.5	19 \pm 0.3	12 \pm 1.5
	0.01	9 \pm 0.6	13 \pm 0.7				
	0.03	10 \pm 2.8	9 \pm 1.5				
	0.10	14 \pm 1.0	7 \pm 0.9				
	0.30	16 \pm 0.7	7 \pm 2.3				
	1.00	11 \pm 3.8	7 \pm 1.2	22 \pm 2.4	13 \pm 1.0	23 \pm 3.0	12 \pm 1.5
	3.30			26 \pm 4.3	10 \pm 2.1	20 \pm 3.4	15 \pm 1.7
	10.00			14 \pm 0.6	9 \pm 2.4	13 \pm 2.0	7 \pm 2.1
	33.00			13 \pm 1.2	15 \pm 2.6	13 \pm 2.5	12 \pm 1.7
	100.00			1 \pm 0.7	13 \pm 2.5	toxic	toxic
	Trial summary	Negative	Negative	Negative	Negative	Negative	Negative
	Positive control	207 \pm 94.2	177 \pm 104.5	356 \pm 21.3	197 \pm 15.3	432 \pm 29.1	122 \pm 43.3

^a The study was performed at Case Western Reserve University. The detailed protocol and these data are presented in Zeiger *et al.* (1987). The high dose was limited by toxicity; 0 $\mu\text{g}/\text{plate}$ dose is the solvent control.

^b Revertants are presented as mean \pm standard error from three plates.

^c 2-Aminoanthracene was used on all strains in the presence of S9. In the absence of metabolic activation, 4-nitro-*o*-phenylenediamine was tested on TA98, sodium azide was tested on TA100 and TA1535, and 9-aminoacridine was tested on TA1537.

TABLE E2
Induction of Sister Chromatid Exchanges in Chinese Hamster Ovary Cells by Benzethonium Chloride^a

Compound	Dose ($\mu\text{g/mL}$)	Total Cells	No. of Chromo- somes	No. of SCEs	SCEs/ Chromo- some	SCEs/ Cell	Hrs in BrdU	Relative Change of SCEs/ Chromosome ^b (%)
-S9								
Summary: Negative								
Distilled water		50	1,047	434	0.41	8.7	26.0	
Mitomycin-C	0.005	25	523	639	1.22	25.6	26.0	194.76
Benzethonium chloride	0.960	50	1,049	458	0.43	9.2	26.0	5.33
	3.000	50	1,050	457	0.43	9.1	26.0	5.00
	9.600	50	1,048	473	0.45	9.5	26.0	8.88
								P=0.115 ^c
+S9								
Summary: Negative								
Distilled water		50	1,043	369	0.35	7.4	26.0	
Cyclophosphamide	1.00	50	1,045	790	0.75	15.8	26.0	113.69
Benzethonium chloride	3.00	50	1,048	347	0.33	6.9	26.0	-6.41
	9.60	50	1,050	359	0.34	7.2	26.0	-3.36
	30.00	50	1,047	367	0.35	7.3	26.0	-0.92
								P=0.495

^a Study performed at Columbia University. A detailed description of the protocol and these data are presented in Galloway *et al* (1987). SCE=sister chromatid exchange; BrdU=bromodeoxyuridine.

^b SCEs/chromosome in treated cells versus SCEs/chromosome in solvent control cells $\times 100$.

^c Significance of relative SCEs/chromosome tested by the linear regression trend test vs. log of the dose

TABLE E3
Induction of Chromosomal Aberrations in Chinese Hamster Ovary Cells by Benzethonium Chloride^a

-S9					+S9				
Dose ($\mu\text{g/mL}$)	Total Cells	No. of Abs	Abs/ Cell	Percent Cells w/Abs	Dose ($\mu\text{g/mL}$)	Total Cells	No. of Abs	Abs/ Cell	Percent Cells w/Abs
Harvest time: 14.0 hours Summary: Negative					Harvest time: 14.0 hours Summary: Negative				
Distilled water					Distilled water				
	100	4	0.04	4.0		100	3	0.03	3.0
Mitomycin-C					Cyclophosphamide				
0.15	50	34	0.68	42.0	15.00	50	16	0.32	28.0
Benzethonium chloride					Benzethonium chloride				
0.96	100	11	0.11	10.0	3.00	100	5	0.05	5.0
3.00	100	10	0.10	10.0	9.60	100	6	0.06	5.0
9.60	100	8	0.08	8.0	30.00	100	6	0.06	6.0
P=0.162					P=0.172 ^b				

^a Study performed at Columbia University. The detailed protocol and these data are presented in Galloway *et al.* (1987).
 Abs=aberrations.

^b Significance of percent cells with aberrations tested by the linear regression trend test vs. log of the dose

Developmental Toxicity Study With Benzethonium Chloride in Rats; #1.

General Information

Reference: Report to Colgate-Palmolive Co.
Report Date: May 5, 1976
Testing Laboratory: Bio/dynamics Inc.
Project No.: 75-1344

Study Design

Test System: Pregnant female Long-Evans rats
Age: Adult
Animal Supplier: Blue Spruce Farms, Altamont, NY
Dose Levels: 0, 1.13, 3.56, 35.6 mg/kg/day
Dose Solution Concentrations: 0, 0.011, 0.036, 0.36 % in water
Dose Volume: 10 ml/kg
Dose Route and Regimen: Oral gavage, daily on GD 6 through GD 15
Number of Animals per Group: 20 dams
Experimental Evaluations: i. Maternal: mortality, clinical signs, body weight, necropsy;
ii. Cesarean section: corpora lutea, implantation sites, resorptions, fetal viability, fetal body weight, fetal crown-rump distance, fetal necropsy;
iii. Fetal examination: gross external, visceral and skeletal.

Results

Maternal: At least 18 dams in each group were pregnant. No mortality or clinical signs of toxicity were observed. A marked decrease in body weight gain during GD 6-15 was observed for dams at the 35.6 mg/kg/day dose level.

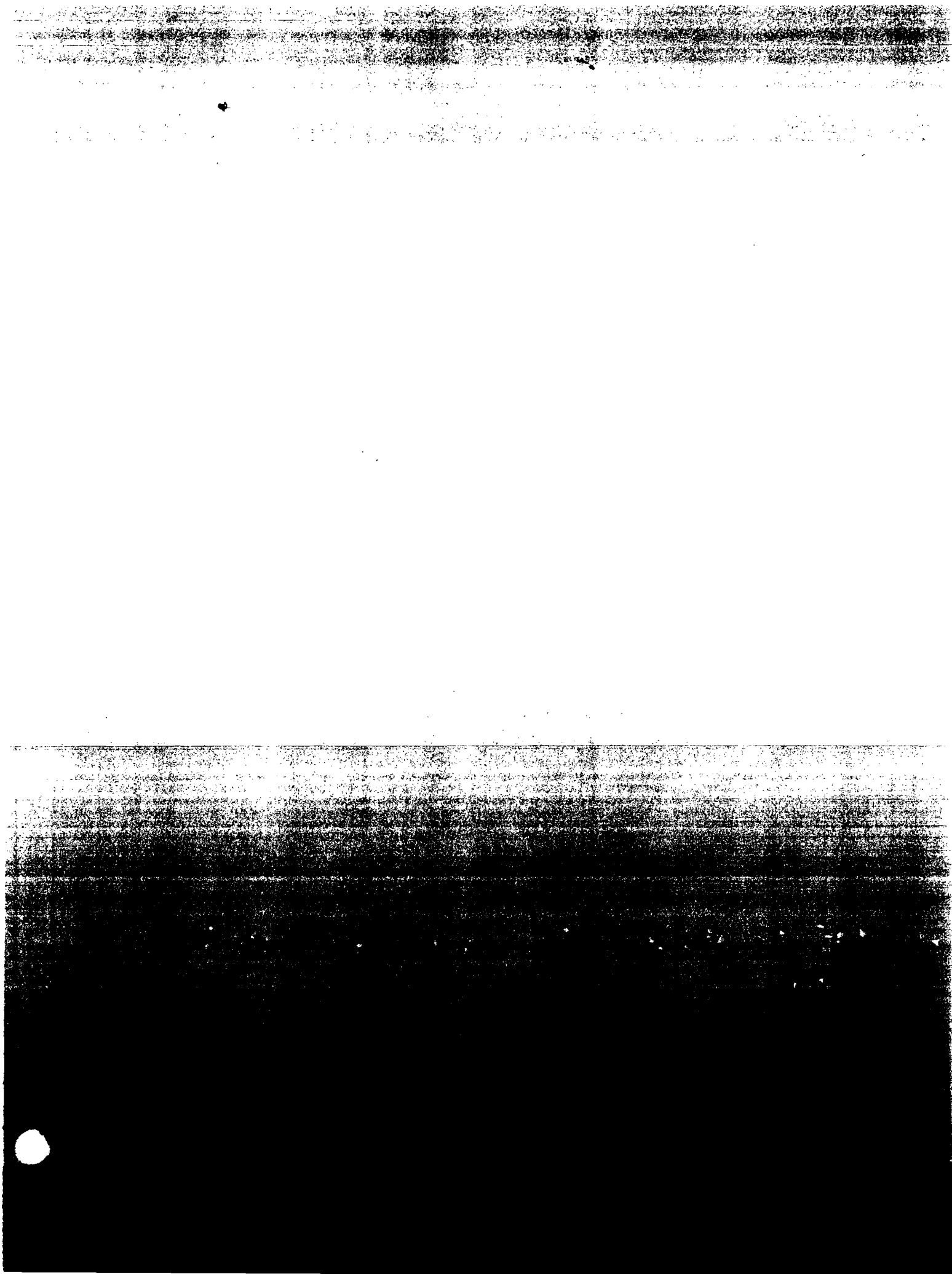
Cesarean Section: Slight decreases in fetal body weight and crown-rump distance and an increase in the incidence of small fetuses were observed in dams at the 35.6 mg/kg/day dose level. These effects were a result of two litters in this group which were composed of abnormally small fetuses. Treatment had no effect on the number of corpora lutea, the number of implantation sites, the number of resorptions, litter size, or fetal viability.

Developmental Toxicity Study With BZC in Rats; #1.

Fetal Examination: An increase in the number of fetuses/litter with skeletal variations (delayed ossifications) were observed for dams in all treatment groups. An increased incidence of fetuses with soft tissue malformations (hydrocephalus) and skeletal malformations (microfetalis, microphthalmia, micromelia) was observed in dams at the 35.6 mg/kg/day dose level. Nearly all of the fetuses with these malformations came from the same litter.

Discussion and Conclusions

Benzethonium chloride, administered at a dose level of 35.6 mg/kg/day to pregnant rats, resulted in maternal toxicity, as evidenced by a marked decrease in maternal body weight gain during the dosing period. Benzethonium chloride at this dose level also resulted in an increase in the incidence of fetuses/litter with skeletal variations. In addition, the incidence of fetuses with skeletal and visceral malformations was increased primarily as a result of effects on one litter. An increase in the incidence of fetuses/litter with skeletal variations (delayed ossification) was observed in animals at benzethonium chloride doses of 3.56 and 1.13 mg/kg/day. It is unclear if the increased incidence of variations observed in the low and intermediate dose groups was related to treatment, based on the following consideration: These variations are commonly observed in untreated animals and the incidence of variations observed in these groups was only slightly above the incidence observed in the control groups in a follow up study conducted at the testing laboratory with benzethonium chloride (Bio/dynamics Project No. 76-1495 A). Under the conditions of the study, the NOEL for maternal toxicity is considered to be 3.56 mg/kg/day. While a clear NOEL for developmental toxicity was not established in this study, it may be as high as 3.56 mg/kg/day.



Developmental Toxicity Study With Benzethonium Chloride in Rats; # 2.

General Information

Reference: Report to Colgate-Palmolive Co.
Report Date: December 2, 1976
Testing Laboratory: Bio/dynamics Inc.
Project No. 76-1495A

Study Design

Test System: Long-Evans rats
Age: Adult
Animal Supplier: Blue Spruce Farms, Altamont, NY
Dose Levels: 0, 0.059, 1.13, 3.56, 35.6 mg/kg/day
Dose Solution Concentrations: 0, 0.00059, 0.011, 0.036, 0.36 % in water (two concurrent control groups were used.)
Dose Volume: 10 ml/kg
Dose Route and Regimen: Oral gavage, daily on GD 6 through GD 15
Number of Animals per Group: 20 to 24 dams
Experimental Evaluations: i. Maternal: mortality, clinical signs, body weight, necropsy;
ii. Cesarean section: corpora lutea, implantation sites, resorptions, fetal viability, fetal body weight, fetal crown-rump distance, fetal necropsy;
iii. Fetal examination: gross external, visceral and skeletal.

Results

Maternal: At least 18 dams in each group were pregnant. No treatment-related mortality or clinical signs of toxicity were observed. A marked decrease in body weight gain during GD 6 to 15 was observed for dams at the 35.6 mg/kg/day dose level.

Cesarean Section: Treatment had no effect on the number of corpora lutea, the number of implantation sites, the number of resorptions, litter size, fetal viability, fetal body weight, or crown-rump distance.

Fetal Examination: An increase in the number of fetuses/litter with skeletal variations (delayed ossifications) was observed for dams at the 35.6 mg/kg/day dose level. Increases in the incidence of skeletal variations were not observed in the lower dose groups.

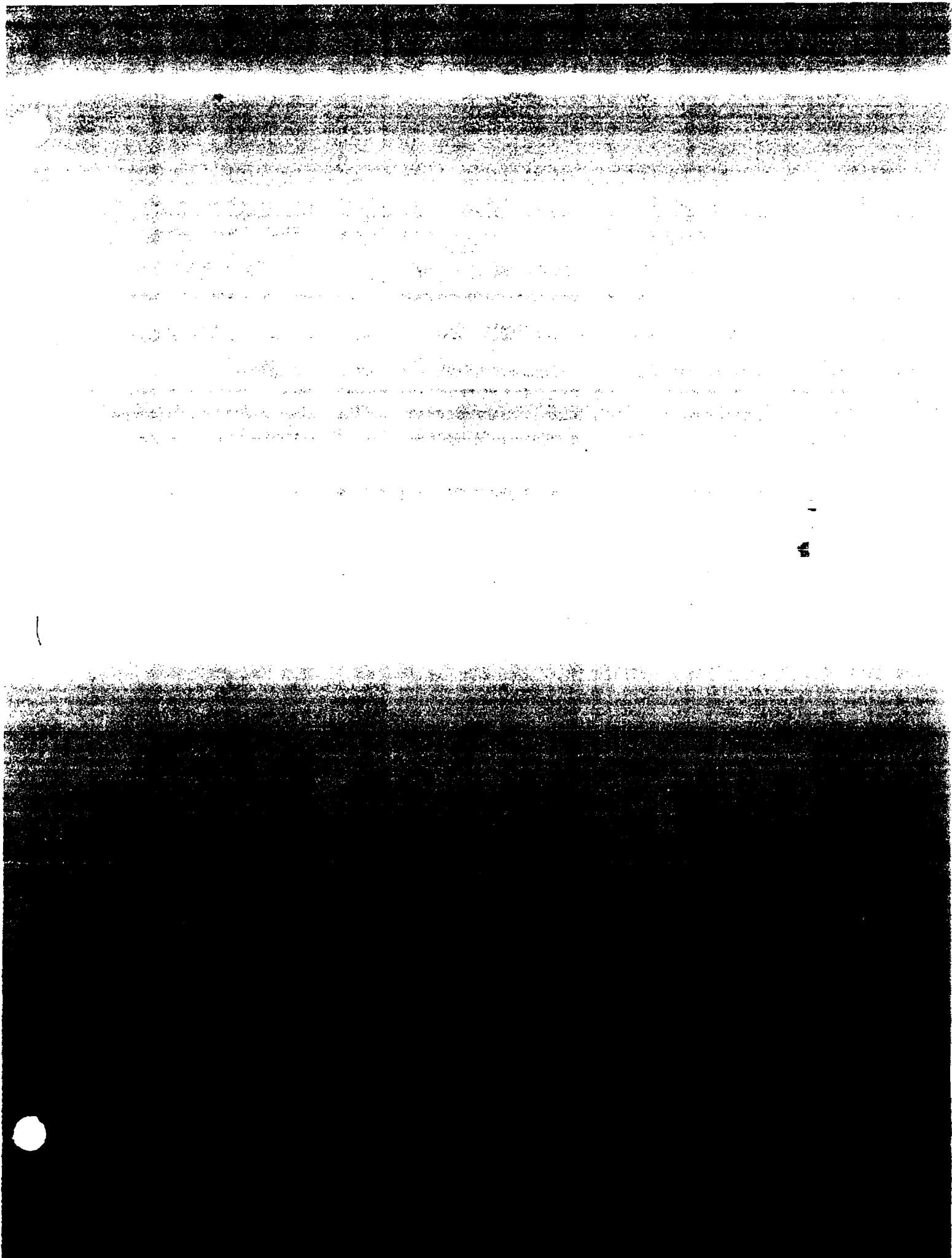
Three litters from the 35.6 mg/kg/day group each had one fetus with wavy ribs. No treatment-related soft tissue malformations or variations were observed.

Comments

The study was conducted in follow-up to developmental toxicity study in rats with benzethonium chloride conducted at the testing laboratory at dose levels of 0, 1.13, 3.56 and 35.6 mg/kg/day (Bio/dynamics Project No. 75-1344). In this previous study, statistically significant increases in the incidence of fetuses/litter with skeletal variations were observed at all dose levels. Also observed in this previous study were maternal toxicity, decreased fetal size and weight, and fetal malformations in dams at the 35.6 mg/kg/day dose level. The current study was performed with two independent control groups and dose levels of 0.059, 1.13, 3.56 and 35.6 mg/kg/day, apparently in an attempt to evaluate the reproducibility of the effects observed in the previous study, establish a clear NOEL for developmental toxicity and better define the range of control incidence for skeletal variations in this test system.

Discussion and Conclusions

Benzethonium chloride administered to pregnant rats at a dose level of 35.6 mg/kg/day resulted in maternal toxicity, as evidenced by a marked decrease in maternal body weight during the dosing period. Benzethonium chloride had no effect on gestational parameters or fetal size or body weight at any dose level. The incidence of fetuses/litter with skeletal variations (delayed ossification) was increased in dams at the 35.6 mg/kg/day dose level. There were no clear treatment-related effects on skeletal or visceral malformations at any dose level. The relationship of the finding of wavy ribs for the three fetuses in the high-dose group to treatment with benzethonium chloride is considered questionable, since the incidence observed in this study was well within the historical control range at the laboratory for wavy ribs. Under the conditions of this study, the NOEL for maternal and developmental toxicity is considered to be 3.56 mg/kg/day.



Perinatal and Postnatal Exposure Study With Benzethonium Chloride in Rats

General Information

Reference: Report to Colgate-Palmolive Co.
Final Report: April 22, 1976
Testing Laboratory: Bio/dynamics Inc.

Study Design

Test System: Pregnant female Long-Evans rats
Age: Adult
Dose Levels: 0, 1.13, 3.56, 35.6 mg/kg/day
Dose Solution Concentrations: 0, 0.011, 0.036, 0.36 % in water
Dose Volume: 10 ml/kg
Dose Route and Regimen: Oral gavage, daily on GD 15 through LD 20
Number of Animals per Group: 20 Dams
Experimental Evaluations: i. Maternal: mortality and clinical signs, daily; body weight on GD 0, 7, 15-21, and LD 0, 7, 14 and 21; necropsy;
ii. Pups: mortality and clinical signs, daily; body weight on postnatal day 0, 4 and 21; necropsy.

Results and Discussion

Maternal: At least 19 dams in each group were pregnant. No treatment-related mortality, clinical signs of toxicity or changes in body weight were observed. There were no test-substance related differences in the number of dams with live litters or gestation length. There were no treatment-related abnormalities observed for dams at necropsy.

Pups: Changes in fetal viability indices and postnatal viability were observed for pups in the intermediate- and high-dose groups. For fetal viability, these included increased resorptions, decreased number of live pups per number of implantation sites, and decreased number of live pups per total number of pups (live and dead). For postnatal viability, survival was decreased during the LD 4 to LD 21 period. The latter changes were due largely to mortality for most or all pups in two litters in each of the intermediate- and high-dose groups. A statistically significant decrease in the number of live pups born per total number of pups (live and dead) was observed for dams in the low-dose group.

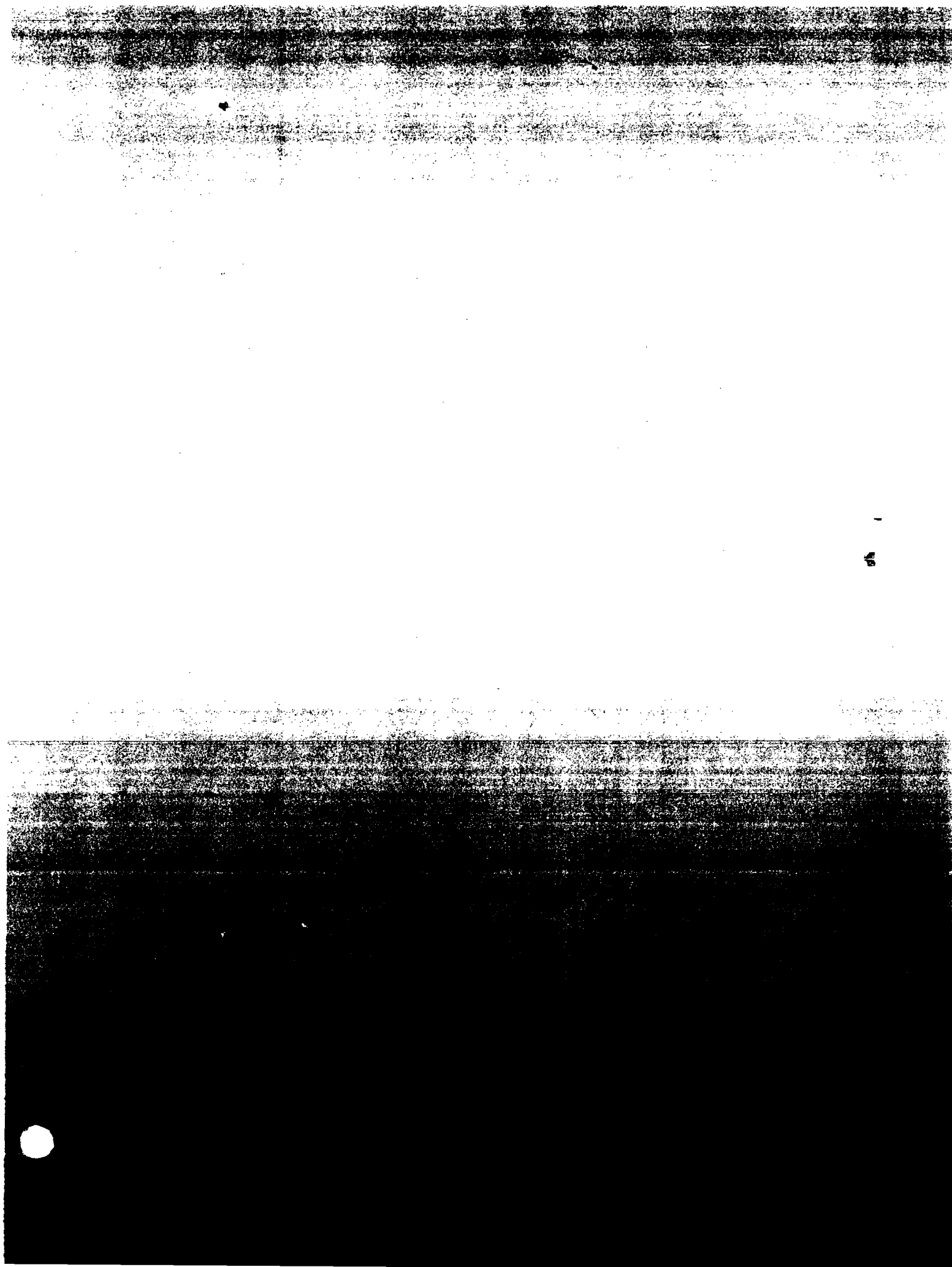
Perinatal and Postnatal Exposure Study With BZC in Rats

Pups continued: This finding was not considered to be related to treatment with benzethonium chloride since the number of live pups per number of implantation sites was unaffected at this dose level. Rather, this is considered to be a spurious statistical finding resulting from a decrease in the number of implantation sites for dams in this group (determined prior to the start of dosing) and a slight numerical decrease in the number of viable pups at this dose level.

There were no treatment-related differences in body weight or sex ratios. At necropsy, the incidence and types of abnormalities observed in the treatment groups were within the range of normal variation for controls and did not reflect treatment-related effects.

Conclusions

Benzethonium chloride administered to female rats from Day 15 of gestation through Day 20 of lactation resulted in decreases in fetal viability and postnatal survival at dose levels of 3.56 and 35.6 mg/kg/day. No effect on mean weight gain for dams through gestation or lactation was observed. No differences from control were seen in the analyses of pregnancy rate, gestation length, mortality, offspring body weights, sex ratios, or necropsy findings. Under the conditions of this study, the NOEL for fetal and postnatal viability is considered to be 1.13 mg/kg/day.



Developmental Toxicity Study With Benzethonium Chloride in Rabbits

General Information

Reference: Report to Colgate-Palmolive Co.
Report Date: July 16, 1976
Testing Laboratory: Bio/dynamics Inc.

Study Design

Test System: Pregnant female New Zealand White rabbits
Age: Adult
Animal Supplier: Perfection Breeders, Douglassville, PA
Dose Levels: 0, 1.13, 3.56, 35.6 mg/kg/day
Dose Solution Concentrations: Not specified
Dose Volume: Not specified
Dose Route and Regimen: Oral gavage, daily on GD 7 through GD 19
Number of Animals per Group: 15 to 27 does
Experimental Evaluations: i. Maternal: mortality, clinical signs, body weight, necropsy;
ii. Cesarean section: corpora lutea, implantation, resorptions, fetal viability, fetal body weight, fetal crown-rump distance;
iii. Fetal examination: gross external, skeletal and necropsy.

Results

Maternal: The numbers of pregnant does per group were 13, 14, 12 and 23 for the 0, 1.13, 3.56 and 35.6 mg/kg/day dose groups, respectively.

Mortality was observed in 0/20, 5/21, 1/15 and 16/27 does in the 0, 1.13, 3.56 and 35.6 mg/kg/day groups, respectively.

Body weight loss from GD 7 to 19 and decreased body weight gain (compared to control) from GD 19 to 30 were observed for does at the 35.6 mg/kg/day dose level. No treatment-related clinical signs of toxicity were noted in the study.

Necropsy findings were observed for does at all three dose levels. However, due to the nonspecific nature of the changes, and the autolysis apparent for animals that died during the study, it is unclear if the necropsy changes noted for animals in the intermediate and low-dose groups are related to treatment.

Cesarean Section: A decrease in the number of viable fetuses and an increase in the number of resorptions were observed in animals at the 35.6 mg/kg/day dose level. Fetal body weight also was decreased in the 35.6 mg/kg/day dose group. No treatment-related changes were observed for the number of implantation sites, fetal crown-rump distance or fetal necropsy findings.

Fetal Examination: There were no gross external findings or skeletal alterations that were attributed to treatment.

Comments

There were a number of spontaneous deaths in this study that occurred prior to the start of dosing. In addition, pneumonia was noted for a few animals in each group that survived until study termination. These findings raise some concern regarding the health status of these animals and their suitability for use in this study.

Discussion and Conclusion

Benzethonium chloride administered to pregnant rabbits at a dose level of 35.6 mg/kg/day resulted in excessive maternal toxicity, as evidenced by a 60% mortality rate and body weight loss during the dosing period. Changes noted at the Cesarean section for animals at this dose level (decreased fetal viability, increased resorptions, decreased fetal body weight) are considered to be a result of maternal toxicity. Mortality also was observed for 5 does in the low-dose group and for one doe in the mid-dose group. The lack of a dose-response relationship for mortality at these dose levels and necropsy findings for some of these animals (fluid in thoracic cavity, ruptured stomach, punctured lung), suggest that some of these deaths were related to the dosing procedure or aspiration of the test substance, rather than a direct effect of treatment.

A clear NOEL for maternal toxicity cannot be determined because of the questionable nature of the necropsy findings observed for animals in the intermediate- and low-dose groups. The NOEL for developmental toxicity is considered to be at least 35.6 mg/kg/day.

Developmental Toxicity Study With Benzethonium Chloride in Rats

General Information

Reference: Report to Lonza Inc.
Report Date: October 26, 1995
Testing Laboratory: Argus Research Laboratories

Study Design

Test System: Pregnant female Crl:CD® (Sprague-Dawley) rats
Age at Start of Test: 79 days
Animal Supplier: Charles River Breeding Laboratories, Portage, MI
Dose Levels: 0, 10, 30, 100, 170 mg/kg/day
Dose Solution Concentrations: 0, 0.1, 0.3, 1.0, 1.7 % in water
Dose Volume: 10 ml/kg
Dose Route and Regimen: Oral gavage, daily on GD 6 through GD 15
Number of Animals per Group: 25 dams
Experimental Evaluations: i. Maternal: mortality, clinical signs, body weight, food consumption, necropsy;
ii. Cesarean section: corpora lutea, implantation sites, resorptions, fetal viability, fetal body weight;
iii. Fetal examination: gross external, visceral and skeletal.

Results

Maternal: At least 24 dams in each group were pregnant. Maternal toxicity was limited to the dams in the high-dose group and included mortality for four animals, clinical signs of toxicity and persistent decreases in body weight and food consumption.

Caesarean Section: Treatment had no effect on the number of corpora lutea, the number of implantation sites, the number of resorptions, litter size, fetal viability or fetal body weight.

Fetal Examination: External, visceral and skeletal examinations did not reveal any treatment-related fetal variations or malformations.

Comments

Dose levels were selected based on the results of a single and repeated dose oral toxicity study in rats and a teratology dose range-finding study in rats. In the preliminary toxicity studies, mortality and clear clinical signs of toxicity were observed at dose levels ≥ 200 mg/kg/day. Effects were not observed at lower dose levels. In the teratology dose range-finding study, a moderate degree of maternal toxicity was observed at the highest dose tested (150 mg/kg/day). No evidence of fetal toxicity was observed at any dose level in the dose range-finding study.

Conclusions

Administration of 170 mg/kg/day of benzethonium chloride to pregnant rats resulted in clear maternal toxicity, as evidenced by mortality, clinical signs of toxicity and decreases in body weight and food consumption. Maternal toxicity was not noted at the lower dose levels. Benzethonium chloride had no effect on gestational parameters or the incidence of variations or malformations. The NOEL in this study for maternal toxicity was 100 mg/kg/day. The NOEL for developmental toxicity was at least 170 mg/kg/day.

Sixteen-Day Dermal Toxicity Study With Benzethonium Chloride in Rats

General Information

Reference: NTP Draft Technical Report
Report Date: June 22, 1994
Testing Laboratory: Battelle Columbus Laboratories

Study Design

Test System: Male and female F344/N rats
Age at Start of Test: 36 to 42 days
Dose Levels: 0, 6.3, 12.5, 25, 50, 100 mg/kg/day.
Dose Solution Concentrations: Males: 0, 0.6, 1.2, 2.4, 4.8, 9.6%
in 95% ethanol USP
Females: 0, 0.4, 0.8, 1.6, 3.2, 6.4 %
in 95% ethanol, USP
Dose Volume: 250 µl
Dose Route and Regimen: Topical, to dorsal interscapular area, unoccluded;
once daily for 16 consecutive days
Number of Animals per Group: Five males and five females
Experimental Evaluations: i. Twice daily observations for mortality and
clinical signs of toxicity; body weight recorded
initially, on day 10 and at the end of the study;
ii. Necropsy;
iii. Organ weight: Brain, heart, kidney, liver, lung,
testis and thymus;
iv. Histopathology of gross lesions and tissue
masses; examination of skin from application
sites and undosed sites.

Results

Mortality and Clinical

Observations: No mortality or clinical signs of systemic toxicity were observed.

Body Weight: Decreased body weight was observed for males and females at the
50 and 100 mg/kg/day dose levels.

Sixteen-Day Dermal Toxicity Study With BZC in Rats

Page 2

Skin Changes: In-life: Information on skin changes noted during the in-life phase of the study was not provided in the laboratory report.

Necropsy: Crusty or red-grey lesions with thickening and hardening of the skin at the site of application were observed for males at dose levels ≥ 25 mg/kg/day and for females at dose levels ≥ 50 mg/kg/day.

Necropsy: Necropsy findings (or lack thereof) for systemic organs and tissues were not discussed in the laboratory report.

Organ Weight: Decreased thymus weights were observed for males and females at 100 mg/kg/day and for females at 50 mg/kg/day.

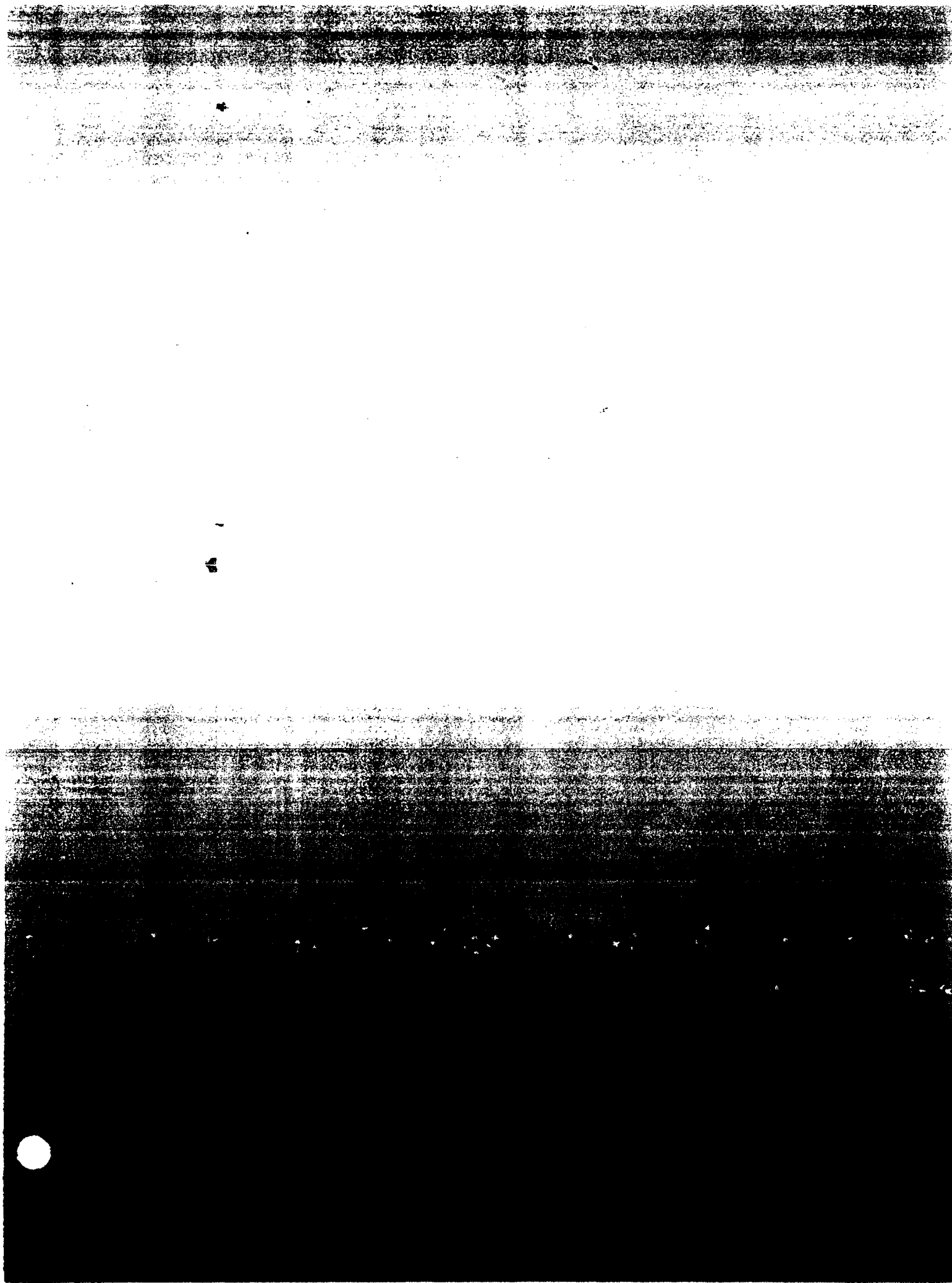
Histopathology: There was a dose-related increase in the incidence and/or severity of animals with epithelial hyperplasia and inflammation at all dose levels.

Mild to moderate chronic inflammation of the dermis and subcutaneous tissues was observed in animals at dose levels ≥ 25 mg/kg/day. Marked ulcerative necrotizing inflammation of the epidermis was observed in animals at dose levels ≥ 50 mg/kg/day.

Conclusions

Dermal application of benzethonium chloride resulted in moderate skin irritation for males and females at the 50 and 100 mg/kg/day dose levels and mild to moderate skin irritation for males and females at the 25 mg/kg/day dose level. In addition, epithelial hyperplasia was observed at the site of application for males and females at all benzethonium chloride dose levels.

Benzethonium chloride resulted in decreased body weight for males and females at 50 and 100 mg/kg/day. Based on the skin lesions observed in this study, 25/mg/kg/day, administered as a 2.5% solution in 95% ethanol, was selected as the high dose level for the 13-week dermal toxicity study in rats.



Thirteen-Week Dermal Toxicity Study With Benzethonium Chloride in Rats

General Information

Reference: NTP Draft Technical Report
Report Date: June 22, 1994
Testing Laboratory: Battelle Columbus Laboratories

Study Design

Test system: Male and female F344/N rats
Age at Start of Test: 28 days
Dose Levels: 0, 1.563, 3.125, 6.25, 12.5, 25 mg/kg/day.
Dose Solution Concentrations: 0, 0.156, 0.31, 0.63, 1.25, 2.5 %
in 95% ethanol, USP.
Dose Volume: \cong 300 μ l.
Dose Route and Regimen: Topical, to dorsal interscapular area, unoccluded;
five applications per week for 13 weeks
Number of Animals per Group: 10 males and 10 females
Experimental Evaluations: i. Twice daily observations for mortality and
clinical signs of toxicity; body weight and
clinical observations recorded weekly;
ii. Necropsy;
iii. Organ weight: Brain, heart, kidney, liver, lung,
testis and thymus;
iv. Complete histopathology on all control and
25 mg/kg/day rats; examination of skin from
application sites and undosed sites from all dose
groups.

Results

Mortality and Clinical

Observations: No mortality or clinical signs of systemic toxicity were observed.

Body Weight: Decreased body weights were observed for males at 25 mg/kg/day.

Skin Changes: In-life: Crusting, thickening and reddening of the skin were observed at the application site for animals at dose levels ≥ 3.125 mg/kg/day.

Necropsy: Assessment of necropsy findings was made based on the review by the NTP Pathology Working Group (PWG). Multiple red foci were observed at the application site for animals at all dose levels. Multiple irregular epidermal crusts and thickened skin was observed for animals at dose levels ≥ 12.5 mg/kg/day.

Necropsy: Necropsy findings (or lack thereof) for systemic organs and tissues were not discussed in the laboratory report.

Organ Weights: There was decreased thymus weight for males and increased kidney weight for females at the 25 mg/kg/day dose level.

Histopathology: There were dose-related increases in the incidence and severity of epithelial hyperplasia at the site of application with chronic inflammation of the dermis at all dose levels. Necrosis and ulceration were observed for animals at dose levels ≥ 3.125 mg/kg/day.

Hypercellularity of bone marrow was observed in male and female rats at the 25 mg/kg/day dose level.

There were no other lesions in this study that suggested an effect of the test substance on systemic organs or tissues.

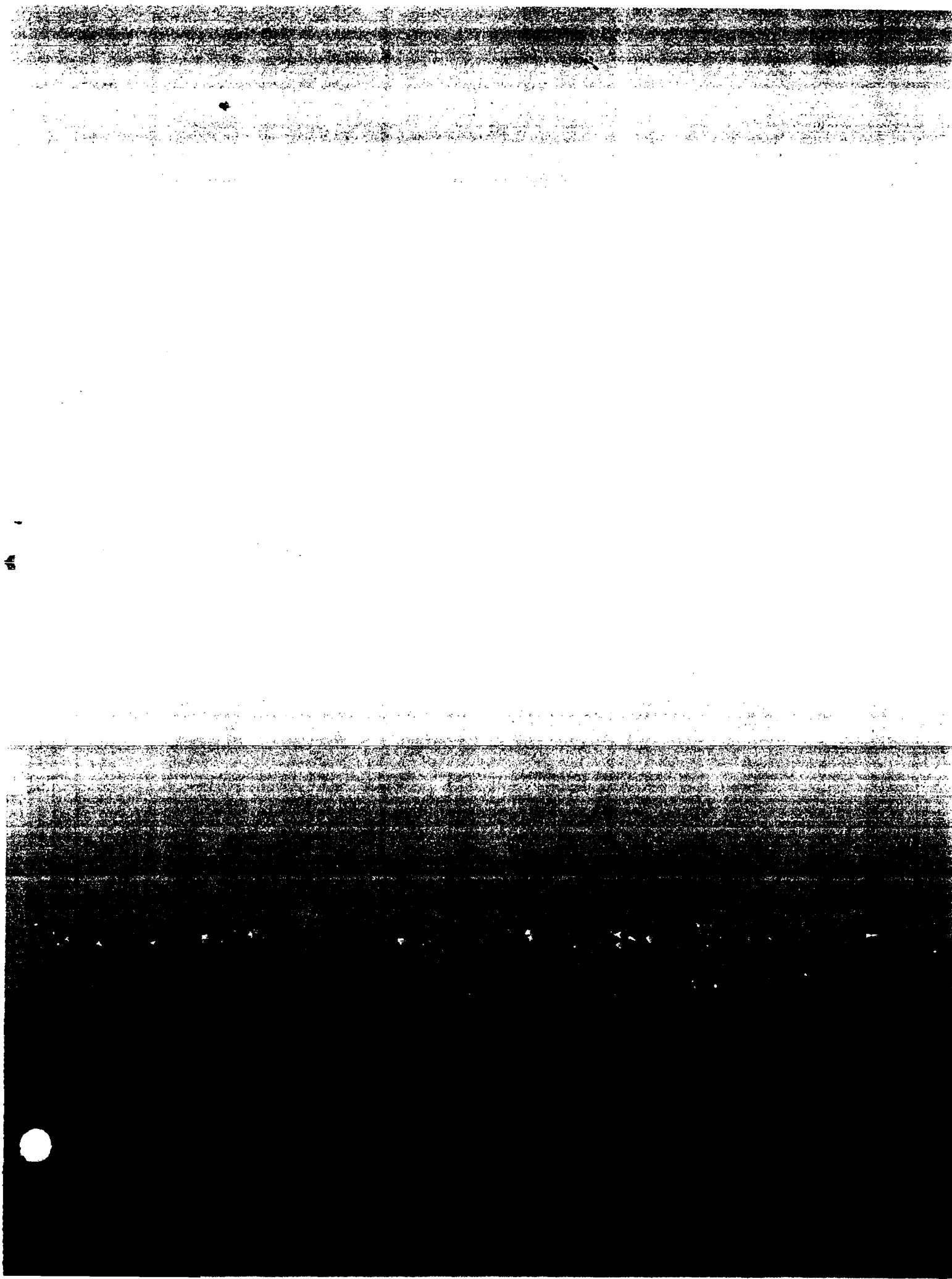
Comments

The pathology results reported by the testing laboratory were reviewed by the NTP PWG. The PWG reclassified and retabulated the data on hyperplastic/proliferative skin lesions to reflect more accurately the changes noted in the study. The PWG also recommended that bone marrow be considered as a secondary target organ in this study. This study summary reflects the conclusions of the PWG.

Discussion and Conclusion

Repeated dermal application of benzethonium chloride for 13-weeks resulted in skin irritation at all dose levels that ranged in severity from mild at the low dose (1.56 mg/kg/day) to marked at the high dose (25 mg/kg/day). Other potentially treatment-related changes observed in animals at the 25 mg/kg/day dose level include decreased body weight for males, increased thymus weight for males, decreased kidney weight for females and an increase in the hypercellularity of the bone marrow for males and females. The changes noted in the organ weights at this dose level were not associated with microscopic changes and are not considered to be biologically significant. It is unclear if the decreased body weight noted for males and the hypercellularity noted for males and females reflect a direct systemic effect of the test substance or an effect secondary to the skin irritation and chronic inflammation noted at the site of application. The lack of anatomic findings in other organs or tissues suggest that these findings are sequelae of the marked skin irritation noted at this dose level.

Based on the skin irritation observed in this study and the evidence for cumulative skin irritation with repeated application (i.e. effects following 13-weeks versus 16-days), 1.5 mg/kg/day, administered as 0.15% to 0.25% solutions in 95% ethanol, was selected as the high dose level for the 2-year dermal toxicity study with benzethonium chloride in rats.



Two-Year Dermal Toxicity Study With Benzethonium Chloride in Rats

General Information

Reference: NTP Draft Technical Report
Report Date: June 22, 1994
Testing Laboratory: Battelle Columbus Laboratories

Study Design

Test System: Male and female F344/N rats
Age at Start of Test: 45 days
Dose Levels: 0, 0.15, 0.5, 1.5 mg/kg/day
Dose Solution Concentrations: Males: 0, 0.025, 0.083, 0.25 %
in 95% ethanol, USP
Females: 0, 0.015, 0.05, 0.15 %
in 95% ethanol, USP
Dose Volume: $\cong 317\mu\text{l}$
Dose Route and Regimen: Topical, to dorsal interscapular area, unoccluded:
five applications per week for 104 weeks
Number of Animals per Group: 60 males and 60 females
Experimental Evaluations: i. Twice daily observations for mortality and
clinical signs of toxicity; body weight recorded
weekly through week 10, once during week 12
and monthly thereafter; clinical observations
recorded monthly;
ii. Necropsy;
iii. Organ weight: Kidney and liver weights for
4 to 9 rats/sex/group at 15-month interim
sacrifice;
iv. Complete histopathology on all control and
1.5 mg/kg/day rats; examination of skin from
application sites and undosed sites from all dose
groups at 15- and 24-month sacrifices.

Results

Mortality and Clinical

Observations: There were no treatment-related changes in survival or clinical
signs of systemic toxicity.

Body weight: There were no treatment-related changes in body weight.

Skin Changes:	In-life: Reddening of the skin was observed for all dose groups. Crusting was observed for males at doses ≥ 0.5 mg/kg/day and for females at a dose level of 1.5 mg/kg/day. Necropsy: Information on skin changes noted at necropsy were not provided in the laboratory report.
Necropsy:	Necropsy findings (or lack thereof) for systemic organs and tissues were not discussed in the laboratory report.
Organ Weights:	No treatment-related changes were observed in liver or kidney weights.
Histopathology- Neoplastic lesions:	There were no neoplastic lesions observed for animals in this study.
Nonneoplastic lesions:	There were no nonneoplastic lesions in this study to indicate an effect of the test substance on systemic organs or tissues. Minimal to moderate dose-related increases in epithelial hyperplasia were observed at the site of application for males and females at dose levels ≥ 0.5 mg/kg/day. Sebaceous gland hyperplasia was sometimes observed in association with more severe cases of epithelial hyperplasia. Epidermal ulceration was observed frequently for females at the 1.5 mg/kg/day dose level and for one male at the 1.5 mg/kg/day dose level. - There were no clear differences between the lesions observed at the 15- and 24-month evaluations.

Conclusions

Repeated dermal application of benzethonium chloride for two years resulted in skin irritation in animals at all dose levels that ranged in severity from minimal in the low-dose (0.15 mg/kg/day) group animals to moderate in the high-dose (1.5 mg/kg/day) group animals. Benzethonium chloride did not result in systemic toxicity or oncogenicity in animals at any dose level. The NOEL in this study for systemic toxicity and oncogenicity was at least 1.5 mg/kg/day.

Sixteen-Day Dermal Toxicity Study With Benzethonium Chloride in Mice

General Information

Reference: NTP Draft Technical Report
Report Date: June 22, 1994
Testing Laboratory: Battelle Columbus Laboratories

Study Design

Test System: Male and female B6C3F₁ mice
Age at Start of Test: 37 to 43 days
Dose Levels: 0, 6.3, 12.5, 25, 50, 100 mg/kg/day
Dose Solution Concentrations: 0, 0.15, 0.3, 0.6, 1.2, 2.41% in 95% ethanol, USP.
Dose Volume: 100 µl
Dose Route and Regimen: Topical, to dorsal interscapular area, unoccluded; twelve applications over a 16-day period
Number of Animals per Group: 5 males and 5 females
Experimental Evaluations: i. Twice daily observations for mortality and clinical signs of toxicity; body weight recorded initially, on day 10 and at the end of the study;
ii. Necropsy;
iii. Organ weight: Brain, heart, kidney, liver, lung, and thymus;
iv. Histopathology of gross lesions and tissue masses; examination of skin from application sites and undosed sites.

Results

Mortality and Clinical Observations: One male at 100 mg/kg/day died on study day four. No other signs of systemic toxicity were observed.

Body Weight: There were no clear treatment-related changes in body weight.

Skin Changes: In-life: Mild crusting, scaling and reddening of the skin at the application site were observed for males at dose levels ≥ 25 mg/kg/day and for females at dose levels ≥ 50 mg/kg/day.

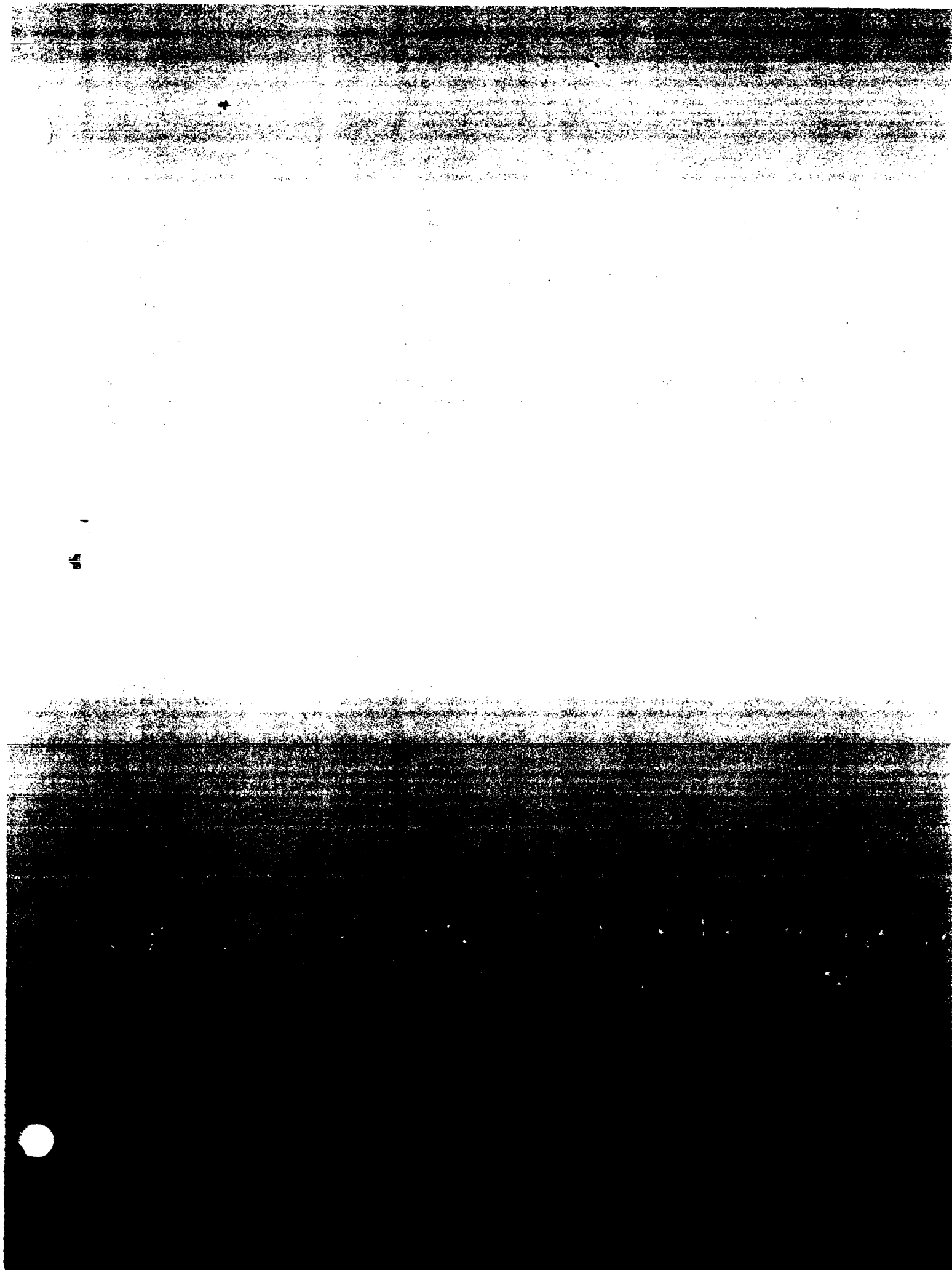
Necropsy: Information on skin changes noted at necropsy were not provided in the laboratory report.

Sixteen-Day Dermal Toxicity Study With BZC in Mice

Necropsy:	Necropsy findings (or lack thereof) for systemic organs and tissues were not discussed in the laboratory report.
Organ Weight:	There was an increase in heart weight for males and females at the 100 mg/kg/day dose level. There was a decrease in thymus weight for females at the 100 mg/kg/day dose level.
Histopathology:	Epithelial hyperplasia was observed with or without inflammation at all dose levels. Mild to moderate necrotizing inflammation of the epidermis was observed for males and females at the 100 mg/kg/day dose level.

Conclusions

Dermal application of benzethonium chloride resulted in moderate skin irritation for males and females at the 100 mg/kg/day dose level, and mild skin irritation for females at the 50 mg/kg/day dose level and males at the 25 and 50 mg/kg/day dose level. In addition, epithelial hyperplasia with inflammation was observed at the site of application at all benzethonium chloride dose levels. Benzethonium chloride did not produce clear evidence of systemic toxicity at any dose level. Based on the skin lesions observed in this study, 25 mg/kg/day, administered as a 0.8% solution in 95% ethanol, was selected as the high dose level for the 13-week dermal toxicity study in mice.



Thirteen-Week Dermal Toxicity Study With Benzethonium Chloride in Mice

General Information

Reference: NTP Draft Technical Report
Report Date: June 22, 1994
Testing Laboratory: Battelle Columbus Laboratories

Study Design

Test System: Male and female B6C3F₁ mice
Age at Start of Test: 27 days
Dose Levels: 0, 1.56, 3.13, 6.25, 12.5, 25 mg/kg/day
Dose Solution Concentrations: 0, 0.05, 0.1, 0.2, 0.4, 0.8 % in 95% ethanol, USP
Dose Volume: \cong 100 μ l
Dose Route and Regimen: Topical, to dorsal interscapular area, unoccluded;
five applications per week for 13 weeks
Number of Animals per Group: 10 males and 10 females
Experimental Evaluations: i. Twice daily observations for mortality and
clinical signs of toxicity; body weight
and clinical observations recorded weekly;
ii. Necropsy;
iii. Organ weight: Brain, heart, kidney, liver, lung,
testis and thymus;
iv. Complete histopathology on all control and
25 mg/kg/day mice; examination of skin from
application sites and undosed sites from all dose
groups.

Results

Mortality and Clinical

Observations: No mortality or clinical signs of systemic toxicity were observed.

Body Weight: Decreased body weights were observed for males at 25
mg/kg/day.

Skin Changes: In-life: Crusting, scaling, thickening and reddening of the skin were observed for males at dose levels ≥ 6.25 mg/kg/day and for females at dose levels ≥ 12.5 mg/kg/day. The incidence and severity of these lesions were not indicated in the laboratory report.

Necropsy: Assessment of necropsy findings was made based on the review by the NTP Pathology Working Group (PWG). Depigmentation was observed for males and females at the 25 mg/kg/day dose level. According to the NTP Pathology Working Group (PWG), this was the only necropsy finding observed in incidences greater than one.

Necropsy: Necropsy findings (or lack thereof) for systemic organs and tissues were not discussed in the laboratory report.

Organ Weight: There were no significant treatment-related changes.

Histopathology: There were no lesions in the study that indicated an effect of the test substance on systemic organs or tissues. A dose-related increase in incidence and/or severity of epithelial hyperplasia at the site of application and chronic inflammation of the dermis was observed for animals at all dose levels. Necrosis was observed for five males and two females in the 25 mg/kg/day group and for one male in each of the 6.25 and 12.5 mg/kg/day groups. Ulceration was observed for one male in the 25 mg/kg/day group.

Comments

The pathology results reported by the testing laboratory were reviewed by an NTP PWG. The PWG reclassified and retabulated the data on hyperplastic/proliferative skin lesions to reflect more accurately the changes noted in the study. This study summary reflects the conclusions of the PWG.

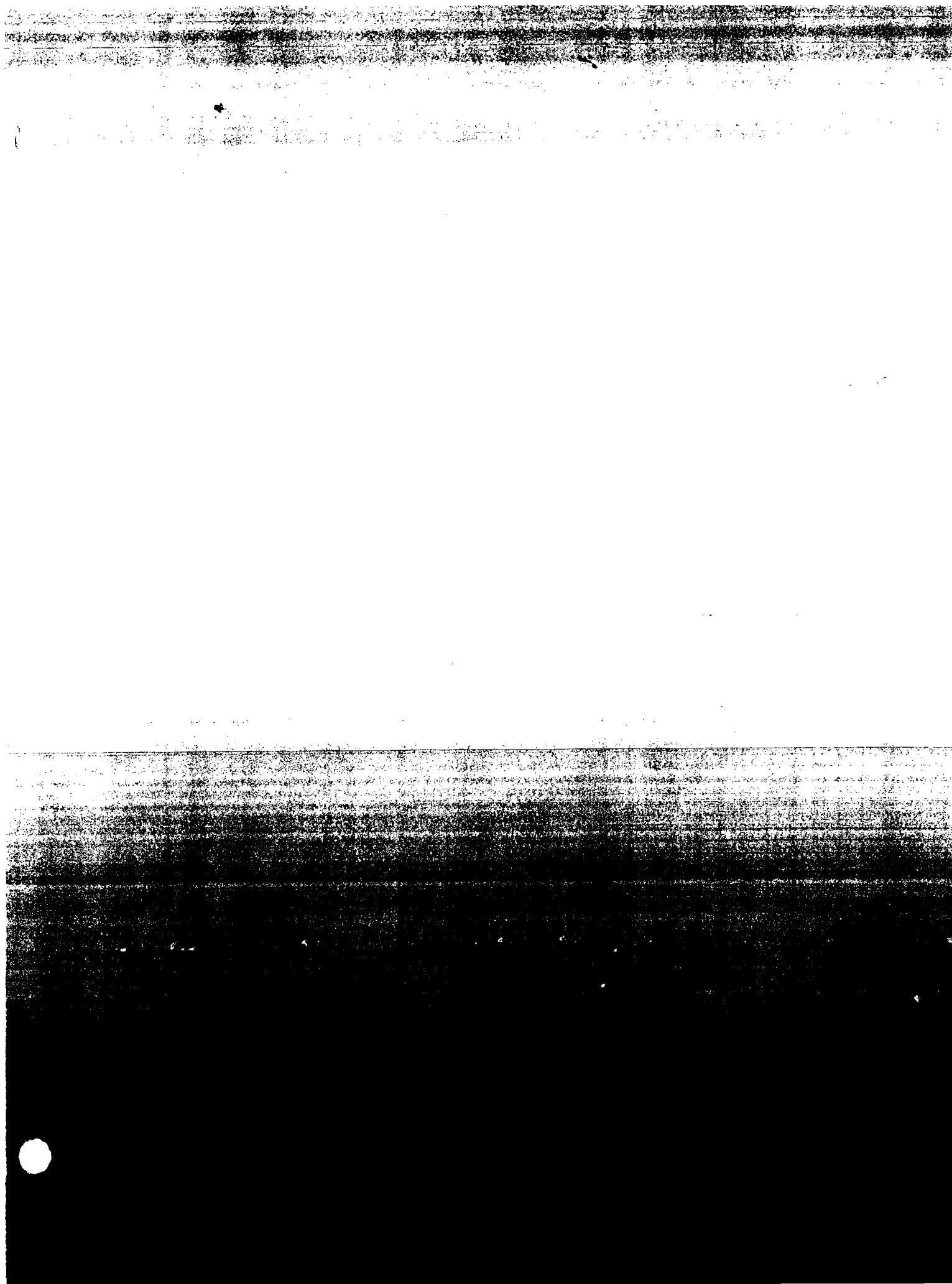
Discussion and Conclusion

Repeated dermal application of benzethonium chloride for 13 weeks resulted in marked skin irritation in animals at the 25 mg/kg/day dose levels and mild skin irritation in animals at the 12.5 and 6.25 mg/kg/day dose levels. In addition, epithelial hyperplasia and inflammation were observed at the site of application at all benzethonium chloride dose levels. There was no evidence of systemic toxicity from benzethonium chloride at any dose level based on gross and histologic examination of a full set of tissues and organs. The small decrease in body weight observed for males in the high-dose group is considered to be secondary to the local irritation

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noted at this level, rather than a direct systemic effect of the test substance. Consequently, the NOEL in this study for systemic toxicity is considered to be at least 25 mg/kg/day.

Based on the skin irritation observed in this study and the evidence for cumulative skin irritation with repeated application (i.e. effects following 13 weeks versus 16 days), 1.5 mg/kg/day, administered as a 0.06% solution in 95% ethanol, was selected as the high dose level for the 2-year dermal toxicity study with benzethonium chloride in mice.



Two-Year Dermal Toxicity Study With Benzethonium Chloride in Mice

General Information

Reference: NTP Draft Technical Report
Report Date: June 22, 1994
Testing Laboratory: Battelle Columbus Laboratories

Study Design

Test System: Male and female B6C3F₁ mice
Age at Start of Test: 40 days
Dose Levels: 0, 0.15, 0.5, 1.5 mg/kg/day..
Dose Solution Concentrations: 0, 0.006, 0.02, 0.06 % in 95% ethanol, USP
Dose Volume: 0.3 ml
Dose Route and Regimen: Topical, to dorsal interscapular area, undosed;
five applications per week for 65 or 104 weeks
Number of Animals per Group: 60 males and 60 females
Experimental Evaluations: i. Twice daily observations for mortality and
clinical signs of toxicity; body weight recorded
weekly through week 10, once during week 12
and monthly thereafter; clinical observations
recorded monthly;
ii. Necropsy;
iii. Organ weight: Kidney and liver weight for
6 to 10 mice/sex/group at 15-month sacrifice;
iv. Complete histopathology on all control and
1.5 mg/kg/day mice; examination of skin from
application sites and undosed sites from all dose
groups at 15- and 24-month sacrifices.

Results

Mortality and Clinical

Observations: There were no treatment-related changes in survival or clinical signs of systemic toxicity.

Body Weight: There were no treatment-related changes in body weight.

Skin Changes: In-life: There was reddening of the skin for the males in all dose groups and for the females in the 0.15 mg/kg/day dose group. Crusting was observed for females at 0.5 mg/kg/day. No information is provided in the report regarding the females in the high-dose group.

Necropsy: Information on skin changes noted at necropsy were not provided in the laboratory report.

Necropsy: Necropsy findings (or lack thereof) for systemic organs and tissues were not discussed in the laboratory report.

Organ Weight: There were no toxicologically significant changes in liver or kidney weights.

**Histopathology-
Neoplastic lesions:** There were no neoplastic lesions observed for animals in this study.

Nonneoplastic lesions: There were no nonneoplastic lesions in this study that indicated an effect of the test substance on systemic organs or tissues.

Dose-related increases in the incidence of animals with epithelial hyperplasia were observed at the site of application for males at the 0.5 mg/kg/day and 1.5 mg/kg/day dose levels and for females at the 1.5 mg/kg/day dose level. Other lesions at the site of application occurred sporadically, or were observed also in the control group, and were not attributed to treatment with the test substance.

There were no clear differences between the lesions noted at the 15- and 24-month sacrifices.

Conclusions

Repeated dermal application of benzethonium chloride for two years resulted in skin irritation that ranged in severity from minimal at the low dose (0.15 mg/kg/day) to mild at the high dose (1.5 mg/kg/day). Benzethonium chloride did not result in systemic toxicity or oncogenicity at any dose level. The NOEL in this study for systemic toxicity and oncogenicity was at least 1.5 mg/kg/day.

CC: M4
CC: GPS

Toxicological Observations on Certain Surface-Active Agents

by J. K. FINNEGAN* and J. B. DIENNA†

THE suitability of wetting agents, detergents and emulsifiers for use in cosmetic products was determined for many years primarily as a function of their physical and chemical properties. Today however, cosmetic chemist will seldom consider for his formulations materials about which he has no basic toxicologic information. He wants to incorporate in his preparations only raw materials of proven safety.

We all agree that it is the responsibility of the manufacturer of the basic agents used in such formulations to provide the chemist with toxicologic data from which a determination can be made of the safety of these agents for the intended use. Since cosmetics are applied to the skin, the first consideration is that of skin irritation or sensitization. Repeated application raises the question of possible absorption into the body through the skin, and consequent effects on the organs. In addition, many products such as face creams, shampoos, and hair dressings can find their way accidentally into the eye. This raises the problem of eye irritation, and possible serious damage to the cornea.

In this study a group of well known anionic, nonionic and cationic synthetic wetting agents, detergents, emulsifiers and antiseptics was examined toxicologically. These products are employed commercially in many cosmetics, toilet goods, and pharmaceuticals such as shampoos, hair rinses, lotions, skin cleansers, hair coloring agents, perfumes, bubble baths, hair waving lotions and antiseptics.

Experimental

Acute Oral Toxicities in Rats:

Although under ordinary conditions of use the most likely methods of contact with these materials would be the skin or mucous membranes, the possibility of accidental ingestion cannot be ignored. Consequently, toxicity studies were done by administering the compounds directly into the stomach of rats by means of a tube and syringe. Ten rats were used at each dosage level and in most cases 4 levels were run. By appropriate choice of doses and suitable statistical treatment of the results a line of regression can be constructed to relate dose to per cent deaths. From this the LD_{50} (dose that would kill 50 per cent of the animals) can be found and its standard errors calculated.

Table I tabulates essential information on the chemical composition of the products tested. Table II presents the oral toxicity results on the compounds tested. It will be noted that the nonionic agents exhibit an intermediate degree of toxicity and toxicity increases as the water solubility increases, reaching a peak at Triton X-100. The anionic agents are less toxic in that their LD_{50} 's are higher. In the case of one of these compounds, Rhotex GS,† it was impossible to introduce enough of the material into the stomach to kill the animals. The cationic

TABLE I
Chemical Composition of Products Tested

Product	Concentration	Chemical Description	Average No. Ethylene Oxide Groups
OPE-1	100%	Octyl phenoxy ethanol	1
OPE-3	100%	Octyl phenoxy polyethoxy ethanol	3
Triton X45	100%	Octyl phenoxy polyethoxy ethanol	5
Triton X114	100%	Octyl phenoxy polyethoxy ethanol	6-8
Triton X100	100%	Octyl phenoxy polyethoxy ethanol	8-10
Triton X102	100%	Octyl phenoxy polyethoxy ethanol	12-13
Triton X200	28% in water	Sodium sulfonated salt of alkyl aryl polyethoxy ethanol	—
Triton X301	20% in water	Sodium sulfated salt of alkyl aryl polyethoxy ethanol	—
Triton 770 Conc.	30% in water and isopropanol	Sodium sulfated salt of alkyl aryl polyethoxy ethanol	—
Triton 771 Conc.	30% in water and isopropanol	Sodium sulfated salt of alkyl aryl polyethoxy ethanol	—
Triton X400	25% in water	20%—stearyl dimethyl benzyl ammonium chloride 5%—stearyl alcohol	—
Rhotex GS	15% in water	Sodium poly acrylate	—
Hyamine 1622	100%	Diisobutyl phenoxy ethoxy ethyl dimethyl benzyl ammonium chloride—monohydrate	—
Hyamine 2389	50% in water	Alkyl (C-18) tolyl methyl trimethyl ammonium chlorides	—

agents exhibit the greatest toxicity, and one of these, Hyamine 2389, differed markedly from the rest in rapidity with which it killed.

On the basis of the known toxicities of potent drugs, however, none of these materials can be called highly toxic.

Percutaneous Toxicities in Rabbits:

The purpose of these experiments was to determine the local

*Medical College of Virginia, Richmond, Va. and †Rohm and Haas Company, Philadelphia.

†Rhotex, Hyamine and Triton are registered trademarks of the Rohm and Haas Company.

TABLE II
Oral Toxicities of Certain Nonionic, Anionic and Cationic Synthetic
Wetting Agents, Detergents, Emulsifiers and Antiseptics in Rats

Class	Compound	LD ₅₀ ± S.E. [cc./kg.]*	Avg. Time of Death (hours)
Nonionic	OPE-1	7.1 ± 0.1	48-72
	OPE-3	4.8 ± 0.2	24-72
	Triton X-45	3.8 ± 0.2	24-96
	Triton X-100	1.80 ± 0.09	<18
	Triton X-102	1.90 ± 0.1	<18
Anionic	Triton X-200	17.5 ± 0.6	<48
	Triton X-301	27.0 ± 0.6	<48
	Triton 770 Conc.	12.5 ± 0.2	<48
	Triton 771 Conc.	13.7 ± 0.4	<48
	Rhotex GS	> 40; no deaths at upper limit of stomach capacity	
Cationic	Triton X-400	4.0 ± 0.1	24-192
	Hyamine 1622	420 ± 25 mg./kg.	24-360
	Hyamine 2389	778 ± 56 mg./kg.	<1

*Doses are given in cc./kg. unless otherwise noted in body of table. All doses are on basis of product as supplied.

effects of these agents on the skin and possible systemic effects following absorption through the skin.

For each compound 6 apparently healthy albino rabbits were selected and the hair clipped from the back and sides from the base of the neck to the hind legs. Two cc. of a solution of the compound was applied to the clipped area of the skin once daily, 5 days a week for 4 weeks. The condition of the skin was watched carefully and the animals were also observed for any signs of systemic toxicity or weight loss. At the termination of the experiment the animals were sacrificed and representative tissues examined histopathologically.

TABLE III
Percutaneous Toxicities of Certain Nonionic, Anionic and Cationic Synthetic
Wetting Agents, Detergents, Emulsifiers and Antiseptics in Rabbits

Class	Compound	Concentration* [%]	Local Effects	Systemic Effects
Nonionic	OPE-1	1 (olive oil)	mild but persistent	none
	OPE-3	1 (olive oil)	mild but persistent	none
	Triton X-100	0.1	none	none
	Triton X-102	0.1	none	none
	Triton X-200	1	none	none
Anionic	Triton X-200	undil.	transient erythema	none
	Triton X-301	1	mild and transient erythema	none
	Triton X-301	undil.	definite erythema	none
	Rhotex GS	undil.	none	none
	Triton X-400	1	mild and transient erythema	none
Cationic	Hyamine 1622	0.1	none	none
	Hyamine 10-X	0.1	none	none
	Hyamine 2389	0.05	none	none

*All doses are on basis of product as supplied.

Table III shows the results of these experiments. In no case were systemic effects apparent and the local erythema that resulted with some of the compounds seems to be referable more to concentration rather than to any particular class of material.

Skin Irritation and Sensitization in Man:

Because these materials are likely to come in contact with the skin repeatedly the following experiments were done:

For each material 50 healthy human subjects were used. A one-quarter inch square of cotton cloth was saturated with the material and placed on the inner surface of the forearm. This was in turn covered with a one inch square of aluminum foil held in place with a two inch square of adhesive tape. Forty-eight hours later the patch was removed and the area inspected for signs of primary irritation. Two weeks after the application of the first patch the procedure was repeated on the other arm to test for possible sensitization caused by the first exposure.

TABLE IV
Skin Irritation and Sensitization Properties of Certain Nonionic,
Anionic and Cationic Synthetic Wetting Agents, Detergents,
Emulsifiers and Antiseptics in Man

Class	Compound	Concentration*	Primary Irritation	Sensitization
Nonionic	OPE-1	undil.	none	4% mild pos.
	OPE-3	undil.	none	none
	Triton X-45	undil.	none	none
	Triton X-100	undil.	none	none
	Triton X-102	undil.	none	none
Anionic	Triton X-200	undil.	none	none
	Triton X-301	undil.	none	none
	Triton 770 Conc.	undil.	none	none
	Triton 771 Conc.	undil.	none	none
	Rhotex GS	undil.	none	none
Cationic	Triton X-400	undil.	definite erythema	none
	Triton X-400	1% solids	none	none
	Hyamine 2389	10% solids	mild erythema	none
	Hyamine 2389	3% solids	none	none

*All doses are on basis of product as supplied, unless otherwise indicated.

As can be seen from Table IV most of the agents produced no primary irritation even in undiluted form. The stronger solutions of the 2 cationic agents tested seemed to be mild to definite irritating agents. As far as sensitization is concerned, only 1 agent, OPE-1, produced a mild positive reaction and then in only 2 of the 50 subjects.

Irritancy on the Rabbit Eye Mucosa:

Since accidental introduction of these materials into the eye is possible even under normal conditions of use, the problem of mucous membrane irritation was intensively studied by 2 techniques.

The first of these we have called the *Irritant Threshold* test. It consists of introducing solutions to be tested into the conjunctival sac of the rabbit eye and after exactly 1 hour observing the treated eye for edema, erythema and increased secretions. In most cases 5 animals were used at each concentration point in the significant range. The threshold concentration was taken as the highest concentration that did not produce signs of irritation in 3 or more of the 5 test animals. These values are given in Table V.

Irritant Thresholds of Certain Nonionic, Anionic and Cationic Synthetic Wetting Agents, Detergents, Emulsifiers and Antiseptics on the Rabbit Eye Mucosa

Class	Compound	Threshold Concentration* (%)
Nonionic	OPE-1	15
	OPE-3	15
	Triton X-45	5
	Triton X-100	0.5
	Triton X-102	1
Anionic	Triton X-200	2
	Triton X-301	5
	Triton 770 Conc.	1
	Triton 771 Conc.	2
	Rhotex GS	2
Cationic	Triton X-400	0.2
	Hyamine 1622	0.03
	Hyamine 10-X	0.03
	Hyamine 2389	0.1

*All doses are on basis of product as supplied.

Although 2 of the nonionic materials, OPE-1 and OPE-3, were of relatively low irritancy, some of the other nonionics were quite toxic. The anionic group were of intermediate toxicity and the cationic agents proved most irritating of all classes, an observation which fits in with our oral toxicity findings.

The other mucous membrane irritation test used in these studies is the *Draize Technique*.^{1, 2} According to this method the reactions or lesions produced in the cornea, iris and conjunctivae are scored separately by means of an arbitrary scale in which approximately 80 per cent of the weight is

TABLE VI
Irritancy of Certain Nonionic, Anionic and Cationic Synthetic Wetting Agents, Detergents, Emulsifiers and Antiseptics on the Rabbit Eye Mucosa Using the Draize Technique

Class	Compound	Highest Tolerated Concentration* (%)	
		Unwashed	Washed
Nonionic	OPE-1	30-50	100
	OPE-3	10-20	100
	Triton X-45	10	50-100
	Triton X-114	5	> 13
	Triton X-100	< 5	> 10
	Triton X-102	5	> 10
	Polyethylene glycol terti-dodecyl ether	< 5	7
Anionic	Triton X-200	3-7	7
	Triton X-301	3-5	13-20
	Triton 770 Conc.	2-5	15-25
	Triton 771 Conc.	5	10
	Rhotex GS	13-20	20-30
	Triton AS-35		
	(No Laundry Batch 1 Sulfate	5-10	20-50
	30% paste) Batch 2	20	30-50
Cationic	Triton X-400	1-2	> 10

*All doses are on basis of product as supplied.

10 rabbits were used. The eyes of these animals were not washed out following the administration of the 0.1 cc. dose of the test substance. Another series of animals (3 used at each concentration) were similarly dosed but the test eye was washed out with 20 cc. of warm water (approximately body temperature) 4 seconds after instillation. Ocular reactions were observed and scored 1, 2, 3, 4 and 7 days after treatment and the scores averaged for each group. Persistence of lesions and their degree at 7 days were taken as the index of irritancy.

Using this technic the compounds listed in Table VI were tested. It will be noted that, in general, the compounds that showed a low irritancy score here also proved to be of a relatively low order of irritancy when tested by the Irritant Threshold test. This finding leads us to suggest that for many purposes (screening etc.) the relatively quicker and less expensive threshold test might produce the information required.

Discussion

It is obvious that no broad generalization as to the toxicological characteristics of all these compounds can be made. We can, however, comment on the characteristics of most of the compounds grouped on the basis of their chemical composition and physical properties:

1. Water insoluble nonionic emulsifiers, OPE-1, OPE-3, Triton X-45.

These materials are all 100% active water insoluble reaction products of alkyl phenol and ethylene oxide. On the basis of the indicated toxicity findings, these products would appear to offer no hazards when used in cosmetics in reasonable amounts.

2. Water soluble nonionic emulsifiers and detergents, Triton X-114, Triton X-100, Triton X-102.

The composition of these products is the same as that of the water insoluble materials just mentioned, with the exception that the hydrophilic part of the molecule has been extended to make the materials water soluble. Again it can be stated that the use of these products would appear to offer no hazard for most cosmetic and toilet products, but due consideration would have to be given to the use of Triton X-100 in shampoos. Note, however, that analogues of this product, Triton X-114 and Triton X-102, having the same basic wetting, emulsifying and detergent properties as Triton X-100, display lower toxicity to the eye, by the Threshold irritation and the Draize tests. These products have respectively shorter and longer water soluble chains than Triton X-100.

3. Water soluble anionic detergents and wetting agents, Triton X-200, Triton X-301, Triton 770 Conc., Triton 771 Conc., Triton AS-35, Rhotex GS.

These compounds, all of similar chemical composition, with the exception of Triton AS-35 and Rhotex GS, show a low order of toxicity to the rat and are intermediate in their potencies as eye irritants. Although some of these agents are irritating when applied repeatedly to the skin of rabbits, they seem innocuous to the skin of man.

4. Cationic wetting agents and bactericides, Triton X-400, Hyamine 1622, Hyamine 10X, Hyamine 2389.

This class of compound in general displays higher values in these tests than the other products studied, particularly where the eye is involved. Special comment can be made on the results of the Draize test on Triton X-400, used widely in cationic hair rinse preparations. The values given for the threshold concentration of severe irritation are in terms of the product as supplied, concentrations considerably in excess of those employed for application to the hair. It is noteworthy however, for all of these products that normal use concentrations in cos-

metic and toilet articles are lower than the indicated irritating concentrations by these tests.

Discussion

DR. LAUFFER: In the Draize test there was a difference between the two batches of sodium lauryl sulphate in the concentration that gave irritation. Could the speaker give us any clue as to the particular difference between the batches that might be responsible for those differences?

DR. FINNEGAN: It is quite possible that the co-author of this paper, Mr. Dienna, could give that information. They were merely submitted to me as No. 1 and No. 2, and that is all I know about them.

MR. DIENNA: The difference between the two batches was that Batch #2 had less stearyl content in the alcohol used in its preparation. The alcohol used in preparing Batch #1 had approximately 6% each of cetyl and stearyl alcohol. Batch #2 had about 11% cetyl and a negligible amount of stearyl alcohol.

DR. SAUL: Do you have any clinical evidence to support the contention that any of these blending agents were absorbed by the skin?

DR. FINNEGAN: We have no clinical evidence to support our

rabbit work. The nearest thing we can get to some clinical information is the effects on man. That is, the irritating and sensitizing properties. I think, though, on the basis of the rather rigorous treatment that we gave the rabbits, that is applying it daily for four weeks, that we might feel rather safe in concluding that these materials are not absorbed to any great extent. Certainly, as far as our histopathologic examination of rabbits' organs and of the skin itself on the area of application is concerned, we found no damage.

DR. POWERS: Triton X-400 I think is accepted to be an unpurified highly unrefined product. Was any effort made to determine whether any of the ingredients in this product were mainly responsible for the toxic effect?

DR. FINNEGAN: We worked with just one sample of the Triton X-400 which was, I think, a twenty-five percent solution of the cationic material in water. We have made no attempts to work on a more highly purified material.

BIBLIOGRAPHY

1. Draize, J. H., G. Woodward and H. O. Calvery, J. Pharmacol. Exptl. Therap. 82, 387-90 (1944).
2. Draize, J. H. and Elaine A. Kelly, Proc. Scient. Sect. Toilet Goods Assoc., No. 17, 1-4 (1952).

"REACTIONS" TO STANDARD PATCH TEST MATERIALS

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Abstract: Twelve compounds tested by closed patch test at recommended concentrations produced numerous "False positive" non-allergic reactions in a panel of 100 volunteers. These results indicate the need for further basic studies to determine appropriate non-irritating concentrations of materials used in biagnostic patch testing for contact allergy.

Key-words: Closed patch testing; False positive; Non-allergic reactions.

Dermatologists daily face perplexing cases of chronic, undiagnosed dermatitis. Contact allergic dermatitis is usually high on the list of differential diagnoses. Currently the main avenues of assistance are a detailed history and diagnostic patch testing. Despite its crudities, no procedure has been developed which provides more reliable information than patch testing. Its limitations include patient discomfort, difficulty in maintaining occlusion, and selection of appropriate concentration and vehicle for the test allergen. But these are trivial troubles compared to the formidable problems of correctly interpreting "positive reactions." The classic allergic contact dermatitis reaction with its clustered vesicles, infiltration and extension beyond the patch is easily identified. The majority of reactions are, however, not archetypical, especially in subjects sensitized weakly to fairly weak allergens. Irritancy responses are often indistinguishable and are the major cause of false positive readings. Fisher's monograph emphasizes these difficulties. His text lists a large series of allergenic chemicals and indicates the concentrations and vehicles in which these can be tested (4). So far as we can determine from his or other lists, proposed concentrations were derived from clinical experience and from a demonstration of non-irritancy in small numbers of "normal" individuals (4,10).

Large-scale patch testing with materials at these concentrations has been conducted by various centers. Lists of the top 20 allergens have been compiled, based on "positive reactions." A major problem recognized by both the North American, European, and International Contact Dermatitis Study Groups is that positive patch tests often do not fit the clinical

circumstance (2, 5, 12). Eliminating exposure to suspected allergens often does not end, or even moderate, dermatitis. Conversely, continued exposure may have no effect. A notable feature of the reports from these groups is the rather high percentage of reactions with certain materials. The NACDG found 11% reacted to 2.5% nickel sulfate, 8% to 0.5% potassium dichromate, 7% to ethylenediamine, 8% to thiomusal, 6% to turpentine, 4% to formalin, and 5% to ammoniated mercury (12). Baer *et al* reported a staggering 22% reaction rate to mercury bichloride, 18% to mercaptobenzothiazole, 13% to paraphenylenediamine, 13% to nickel sulfate, and 12% to formalin (1). These test populations are a highly selected group of clinic patients with a persistent dermatitis which in itself may influence the reactions. One form of this heightened non-specific reactivity is commonly referred to as the "angry back" syndrome. These difficulties have also been stressed by Kligman and Epstein (8). They were forced to face the problem of interpretation when testing the same materials on normal individuals on the East and West Coasts yielded divergent results. Substances not thought to be allergenic often gave positive reactions in the West are negative ones in the East. The denouement of this was that technicians, even highly skilled and experienced ones, were recording non-allergic reactions as "positive." The principal source of false positive reactions was primary irritation, miliaria rubra, and chemical folliculitis. At times it may be necessary to resort to biopsy to settle the issue.

Another major yet rarely discussed or investigated factor in the production of confusing non-allergic reactions is the enormous individual variability in susceptibility to irritants. In our ongoing studies of the irritant potential of various chemicals such as soaps and solvents, we are repeatedly struck at the extreme reactivity of many subjects and the contrasting indifference of others. For example, 50% ammonium hydroxide will produce an intraepidermal blister in 10 or 12 minutes in most subjects. Occasional subjects blister in 2 minutes, while others show nothing after 30

minutes (6). In general, light-skinned persons of Celtic background have more irritable skins.

The finding of non-relevant positive patch tests, coupled with our own increasing awareness of the extreme variability in the reactivity of skin to irritants, led us to examine the battery of allergens recommended for diagnostic patch testing. Our aim was to determine the prevalence of non-allergic "positive" reactions in a normal population of adult males free of any skin disease.

SUBJECTS

One hundred white male prisoners aged 21 to 50 served as volunteers. None had any evidence of skin disease and none were receiving any medication.

Test Materials

Twelve materials were selected from Fisher's original list (3). Forty-eight hour occlusive patches were applied to the upper back, with one cm² of Webril® loaded with 0.1 ml of the test material. The patch was sealed to the skin under an impermeable dressing of a 2 cm square of Saran Wrap® covered by overlying strips of plastic tape (3M Blendern®). The patches were removed after 48 hours and the sites read immediately, at 24, 48, and 72 hours later.

Criteria of Non-Allergic Reactions

We decided that a reaction was non-allergic when there was only redness without visiculation or infiltration, declining in intensity 24 hours after removal, non-itching, and not spreading beyond the patch. In doubtful cases, we repeated the patches on the forearms (a less sensitive area); 3 mm punch biopsies were obtained to rule out allergy in cases unsettled by repeat patch testing.

RESULTS

The results are summarized in Table 1. Some substances produced large numbers of non-allergic reactions, e.g., 22 of 100 reacted to 1% cantharidin, 61 reacted to 90% dimethyl sulfoxide, 92 reacted to 5% sodium lauryl sulfate, and 51 to Hyamine 1622. Each of these is known to be an irritant rather than an allergen. Formalin is a well known sensitizer; 16% of the panel gave reactions that were judged to be non-allergic and this was verified histologically in 4 cases. With nickel

Table 1. False positive patch test reactions

Compound	Reaction rate
Aerosol OT (dioctylester of sodium sulfosuccinic acid), 5% aqueous	4/100
Cantharidin, 1% in ethanol	22/96
Olive oil, 25% in coster oil	3/100
Dimethyl sulfoxide, 90% aqueous	61/100
Sodium lauryl sulfate, 5% aqueous	92/100
Formalin, 5% aqueous	16/100
Hyamine 1622 (p-diisobutyl-phenoxyethoxyethyl-dimethylbenzylammonium chloride), 5% aqueous	51/100
Kerosene, 60% in olive oil	11/100
Nickel sulfate, 5% aqueous	8/96
Oleyl alcohol, 30% in petrolatum	13/100
Salicylic acid, 5% in petrolatum	4/100
Coal tar, 5% in petrolatum	5/96

sulfate, a leading allergen in most series, 8 of 96 responses were false positives; biopsy in 3 showed the findings of irritation (8). The lowest false reaction rate was 3% to olive oil.

DISCUSSION

These results highlight the need for further study of appropriate concentrations of materials for patch testing. The investigator faces the dilemma of choosing, on the one hand, a concentration so high that it is potentially irritating and, on the other, so low that there is insufficient penetration to evoke a response except in strongly sensitized subjects. The ideal is to select the highest non-irritating concentration. The latter cannot be established on a few nearby "normals," an advice which is simply too heterogeneous. Besides there are the variables of race, sex, age, skin type, and the patch itself. Those who do much patch testing are not unaware of the problem. Fisher himself has changed his recommendation for patch testing with nickel sulfate from 5% to 2.5% salicylic acid has been lowered from 5% to 1%, and formalin from 5% to 2%. In our opinion, the data of Baer *et al* includes a considerable number of false "positive" reactions. Patrick, Panzer & Derbes noted "only a 7%" reaction rate to 20% neomycin in normal individuals (11). We would view this rate as another example of over-reading patch tests. Recently in a survey of over 2,000 subjects we detected only 2 bona fide cases of contact allergy, while 37 non-allergic reactions were clearly identified (9). Moreover, we demonstrated non-allergic reaction to one brand of hydrophilic ointment

which turned out to contain Iden and Schroeter noted fre-
tions in 700 patients tested
materials, most frequently
hexachlorophene (11%), and

"Reactions" with patch test:
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patch test reactions

	Reaction rate
of sodium sulfosuccinic	4/100
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il	3/100
aqueous	61/100
aqueous	92/100
	16/100
utyl-phenoxyethoxyethyl-	
m chloride), 5% aqueous	51/100
l	11/100
is	8/96
rolatum	13/100
latum	4/100
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DISCUSSION

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Iden and Schroeter noted frequent non-allergic reac-
tions in 700 patients tested with a battery of 14
materials, most frequently mercuric acetate (17%),
hexachlorophene (11%), and thiomerosal (10%) (7).

"Reactions" with patch testing can be caused by a
variety of non-immunological mechanisms. The com-
mon problems of miliaria, pyoderma and transient
erythema from pressure are generally well recognized.
Other less appreciated but extremely important causes
of false positive, non-allergic reactions include several
possibilities. First is the so-called "angry back" syn-
drome in which a patient with a strong contact allergy
to one allergen will simultaneously develop non-
allergic reactions to other materials. The non-all-
ergic nature of these "reactions" are verified by repeat
patch testing with individual agents. Translocation of
an allergen from one patch to nearby dressings can also
result in multiple "positive reactions" which clearly
appear allergic in nature. Positive patch test reactions
which are clinically allergic in nature and immediately
adjacent to one another should be repeated to rule out
this possibility. Another potential source of false
positive reactions is the phenomenon of para-allergy
(8). In this reaction, there is hematogenous spread of
an allergen from one site and localized at another site,
where a lowlevel irritant reaction calls for repeat,
individual testing.

In this paper we have added another worry for the
use of patch testing, namely contact irritant reactions
to standard recommended concentrations. Clearly
more basic information is needed on the potential
irritancy of the currently recommended concentra-
tions for known allergens. The enormous range of
individual susceptibility to irritation make this infor-
mation an obvious requirement for avoidance of
confusing non-allergic reactions.

REFERENCES

1. Baer, R. L., Ramsey, D. L. & Biondi, E.: The most common contact allergens. *Arch Dermatol* 108: 74, 1973.
2. Bandmann, H.-J. et al.: Dermatitis from applied medica-
ments. *Arch Dermatol* 106: 335, 1972.
3. Fisher, A. F.: Contact Dermatitis. Lea & Febiger,
Philadelphia, 1965.
4. Fisher, A. F.: Contact Dermatitis. Lea & Febiger,
Philadelphia, 1973.
5. Fregert, S. et al.: Epidemiology of contact dermatitis.
Trans St Johns Dermatol Soc 55:17, 1969.
6. Frosch, P. J. & Kligman, A. M.: Rapid blister formation
in human skin with ammonium hydroxide. *Brit J Derma-
tol* 96:461, 1977.
7. Iden, E. & Schroeter, A. L.: The vehicle tray revisited:
The use of the vehicle tray in assessing allergic contact
dermatitis by a 24 hour application method. *Contact
Dermatitis* 3:122, 1977.
8. Kligman, A. M. & Epstein, W.: Updating the maximiza-
tion test for identifying contact allergens. *Contact Der-
matitis* 1:231, 1975.
9. Leyden, J. J. & Kligman, A. M.: Studies in contact
dermatitis to neomycin sulfate. In press (*J Am Med
Assoc*).
10. Magnusson, B.: Patch testing. IN Sunlight and Man.
(eds.) Fitzpatrick, T. B., Pathak, M. A., Harber, L. C.,
Seiji, M. & Kuhita, A. Univ. of Tokyo Press, 1974.
11. Patrick, J., Panzer, J. D. & Derbes, V. J.: Neomycin
Sensitivity in the normal (nonatopic) individual. *Arch
Dermatol* 102:532, 1970.
12. Rudner, E. J. et al.: Epidemiology of contact dermatitis
in North America: 1972. *Arch Dermatol* 108:537, 1973.

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CLINICAL AND BACTERIOLOGICAL STUDY OF PHEMEROL AS A SKIN ANTISEPTIC

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The evaluation of skin antiseptics is a difficult problem in which to arrive at any forthright conclusions. These difficulties are magnified by the variations in the techniques employed by different investigators, by the selection of bacterial cultures for study, by the interference of bacteria from skin glands, and by the addition of oils and fluids. Because of the variations in the use of skin antiseptics, it is quite difficult for any one agent will be found to be superior to others in *all* categories of use. The technique of investigation, therefore, by which an antiseptic can be shown to be supe-

rior in this study. One half of the abdomen was then treated with the antiseptic studied, the soap and alcohol side being used for comparison; as the study progressed, the two sides of the abdomen were painted with different antiseptics for comparison. The accompanying diagram illustrates this technique. Small culture plates¹ containing blood agar were placed on the abdomen as soon as the antiseptic was dry. They were removed at half-hour intervals and incubated at 37 degrees C for 48 hours. The number of colonies was counted and the bacteria partially identified by subculture and stained smear.

No attempt was made to study all of the commercial antiseptics which are available. We selected members of the following groups: cresol-mercurials (Novak's solution and mercresin), the halogens (iodine), organic mercurials (merthiolate), and detergents (phemerol). It is impossible to set up exact parallels of concentration in these vastly different antiseptics. We have used the stock solutions in each case as recommended by the author and manufacturer. (Novak's solution—stock: tincture of mercresin—1:1000; tincture of merthiolate—1:1000; tincture of iodine—7 per cent; aqueous phemerol—1:500; and tincture of phemerol—1:500).

We also studied the effect of increased body temperature and sweating on the bacterial action of phemerol and made observations on skin irritation, clinical morbidity, and wound healing.

RESULTS

Using this technique of clinical bacteriology, we have studied a total of 1020 plates on 102 patients and have subcultured and identified over 50,000 bacterial colonies. The clinical studies were made on 300 consecutive obstet-

ric patients. It has been arranged to parallel the technique of surgery for the preparation of the abdomen. We believe this is a practical and important method of comparing the clinical use of skin antiseptics.

The purpose of this paper to report clinical and bacteriological studies on an antiseptic, phemerol, in comparison with the detergents, the cresol-mercurials, and the organic mercurials. This antiseptic is a tertiary-octyl-ammonium salt of dimethyl-benzyl-ammonium chloride. It is a detergent. This antiseptic is used in the obstetric and gynecologic wards, as a modification of the technique of Novak and Hall in the preparation of the abdomen.

The technique of this study is as follows: The routine technique of this study is as follows: The preparation of the abdomen for surgery. The abdomen is scrubbed with soap and gauze, rinsed with alcohol, and painted with an antiseptic. The patient is then prepared to have a base line for comparison. The same preliminary cleans-

ing is done in the obstetric and gynecologic wards. This study was made possible by a grant from the University of Nebraska.

¹Small aluminum plates, 16 sq. cm. surface—Eimer and Amen, N. Y.

TABLE I
Number of colonies per plate

Patient	Tincture of phemerol					Tincture of soap and alcohol			
	$\frac{1}{2}$ hr.	1 hr.	1 $\frac{1}{2}$ hr.	2 hr.	2 $\frac{1}{2}$ hr.	$\frac{1}{2}$ hr.	1 hr.	1 $\frac{1}{2}$ hr.	2 hr.
1	3	0	18	18	20	20	35	209	100
2	1	0	4	0	3	0	14	6	0
3	8	1	26	19		24	47	0	0
4	0	0	0	3	1	3	42	0	0
5	0	8	9	2	3	4	8	0	0
6	3	17	1	12	27	5	6	0	0
7	3	3	12	5	8	33	11	0	0
8	0	10	14	47	20	0	0	0	0
9	3	0	15	2	0	23	15	0	0
10	4	5	7	18	23	0	0	0	0
11	0	0	1	0	4	0	0	0	0
12	0	1	3	1	0	1	0	0	0
13	2	2	1	1	0	3	0	0	0
14	1	4	1	2	3	3	0	0	0
15	1	0	0	0	6	1	0	0	0
16	3	8	3	11	4	5	0	0	0
17	1	0	0	0	1	0	0	0	0
18	4	2	0	0	4	2	0	0	0
19	0	2	1	4	2	0	0	0	0
20	2	0	0	0	2	16	0	0	0
21	2	0	5	9	3	0	0	0	0
22	5	0	8	4	6	19	0	0	0
Total colonies	46	106	129	148	192	166	100	209	100

*Plates contaminated

rical and 100 surgical cases. In the tables will be found the results obtained in these studies.

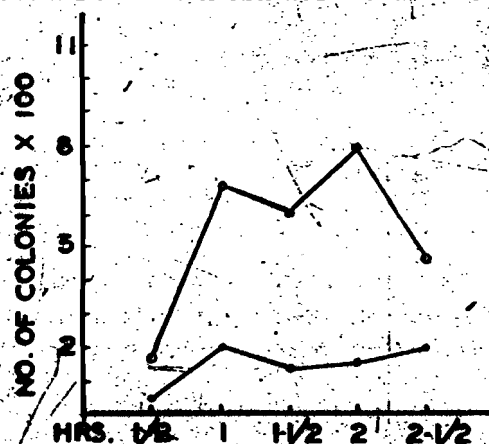


Fig. 1. Tincture of phemerol, —•—; tincture of soap and alcohol ---o---.

In Table II, the results of the green, aqueous phemerol and merol are shown. In many cases, the abdominal bacteriostasis is demonstrated in Figure 2.

Table III shows the results in which tincture of aqueous phemerol and merol is compared with tincture of parent in the study of the relative bacteriostatic power of phemerol and merol.

Table IV shows the results of phemerol and merol in almost 100 cases of Novalis.

Number of colonies per plate

Patient	Tincture of phemerol					Novak's solution				
	$\frac{1}{8}$ hr.	1 hr.	$1\frac{1}{4}$ hr.	2 hr.	$2\frac{1}{2}$ hr.	$\frac{1}{8}$ hr.	1 hr.	$1\frac{1}{4}$ hr.	2 hr.	$2\frac{1}{2}$ hr.
1	0	5	6	6	9	2	9	28	48	100
2	1	2	7	4	10	0	5	45	25	20
3	0	0	0	1	1	1	3	0	3	0
4	1	3	3	2	1	1	48	9	21	0
5	0	3	4	2	4	1	4	3	6	0
6	0	0	0	0	1	1	4	0	0	0
7	1	1	4	0	2	1	0	0	0	0
8	0	0	0	0	0	0	1	0	0	0
9	0	9	3	0	2	0	0	0	0	0
10	1	1	0	0	0	1	0	0	0	0
11	0	0	0	0	1	0	0	0	0	0
12	1	1	0	0	1	0	0	0	0	0
13	2	1	3	1	3	1	0	0	0	0
14	1	0	0	1	0	0	0	0	0	0
15	2	0	0	7	5	1	0	0	0	0
16	1	0	0	0	3	0	0	0	0	0
17	0	1	4	1	2	4	0	0	0	0
18	1	1	0	0	0	1	0	0	0	0
19	0	0	0	1	1	0	0	0	0	0
20	2	0	1	2	2	1	0	0	0	0
Total colonies	14	28	35	28	47	19	120	80	100	100

TABLE IV
Number of colonies per plate

Patient	Tincture of phemerol					Novak's solution				
	$\frac{1}{8}$ hr.	1 hr.	$1\frac{1}{4}$ hr.	2 hr.	$2\frac{1}{2}$ hr.	$\frac{1}{8}$ hr.	1 hr.	$1\frac{1}{4}$ hr.	2 hr.	$2\frac{1}{2}$ hr.
1	0	1	3	1	11	0	0	0	0	0
2	0	0	2	0	12	0	0	0	0	0
3	0	0	0	1	1	0	0	0	0	0
4	1	2	4	1	5	0	0	0	0	0
5	0	0	5	0	0	0	0	0	0	0
6	0	3	0	1	3	0	0	0	0	0
7	0	1	0	0	8	0	0	0	0	0
8	0	1	0	0	1	0	0	0	0	0
9	0	0	1	14	0	0	0	0	0	0
10	0	1	1	2	0	0	0	0	0	0
11	0	1	3	5	6	0	0	0	0	0
12	0	0	0	2	1	0	0	0	0	0
Total colonies	1	8	19	27	37	0	0	0	0	0

*Plates contaminated

TABLE V
Number of colonies per plate

	Tincture of phemerol					Tincture of merthiolate				
	1/2 hr.	1 hr.	1 1/2 hr.	2 hr.	2 1/2 hr.	1/2 hr.	1 hr.	1 1/2 hr.	2 hr.	2 1/2 hr.
1	2	1	6	2	1	10	11	13	25	105*
2	0	0	0	1	5	0	1	1	5	4
3	0	0	0	1	0	0	2	4	5	11
4	0	0	0	4	1	0	2	2	6	5
5	0	0	0	0	0	11	4	8	13	8
6	0	0	0	2	3	2	0	6	21	0
7	0	0	1	1	2	5	4	6	2	4
8	0	1	3	2	2	15	5	3	7	7
9	1	1	1	3	4	6	10	5	14	10
10	3	3	11	16	18	49	39	48	98	163

identified: Streptococcus, Staphylococcus aureus and albus, and sarcina were uniformly present. While no statement can be made regarding a decrease in any specific organisms, it is remarkable that only three colonies of streptococci were found on all these plates and these were on 2 patients (Case 9, Table IV). We found less staphylococcus aureus than albus, and the latter was rather infrequently present. Several other organisms were identified, chief of which were bacilli, staphylococci, gram-negative organisms of the colon group, and pseudomonas. Pseudomonas was found. All of these organisms were quite uniformly scattered

throughout the plates and did not vary significantly with the various antiseptics studied.

Concomitantly with these studies, observations were made to determine skin sensitivity or other toxic reactions which might be attributed to phemerol when employed as an antiseptic. The skin of these patients was carefully observed for several days following its application, and no evidence of irritation, desquamation, or other reactions were noted. Phemerol was used routinely on 300 consecutive obstetric deliveries and 100 surgical cases without evidence of skin irritation. This is in contrast to high incidence of skin irritation from Novak's solution (15%) which we have

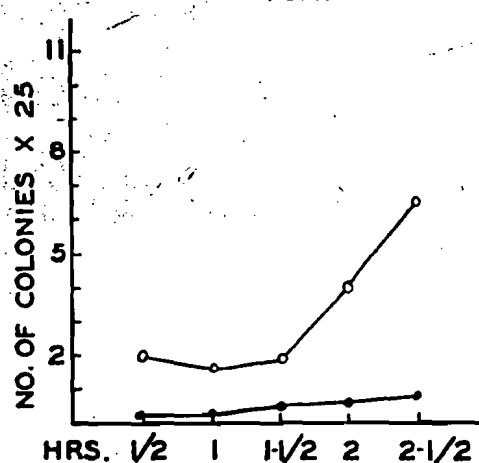
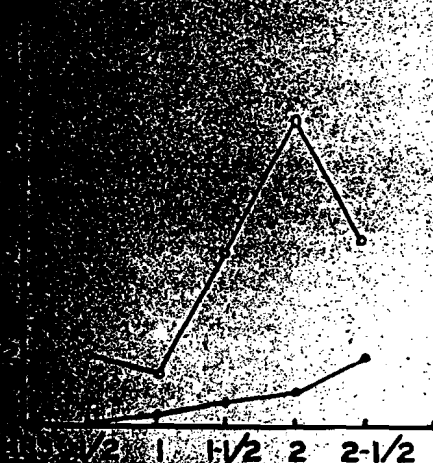


Fig. 4. Tincture of phemerol ———; tincture of merthiolate ○—○—○.

TABLE VI
Number of colonies per plate

Patient	Tincture of phemerol					Tincture of iodine†			
	½ hr.	1 hr.	1½ hr.	2 hr.	2½ hr.	½ hr.	1 hr.	1½ hr.	2 hr.
1	1	2	10	5	3	4	4	2	0
2	0	1	1	0	0	1	1	0	0
3	0	0	2	0	0	0	0	0	0
4	0	2	3	10	11	0	3	0	0
5	3	1	3	5	1	1	2	0	0
6	0	1	4	1	4	1	2	0	0
7	0	1	3	0	0	0	0	0	0
8	0	0	0	1	0	0	0	0	0
9	0	0	0	0	0	0	0	0	0
10	0	1	0	1	3*	0	0	0	0
11	6	0	0	0	2	0	0	0	0
12	0	0	3	1	1	0	0	0	0
Total colonies	10	9	29	24	25	7	23	0	0

†Washed off with 70% alcohol
*Plates contaminated

TABLE VII
Number of colonies per plate

Patient	Tincture of phemerol—normal thigh					Tincture of iodine			
	½ hr.	1 hr.	1½ hr.	2 hr.	2½ hr.	½ hr.	1 hr.	1½ hr.	2 hr.
1	0	1	1	0	0	0	0	0	0
2	0	0	0	1	0	0	0	0	0
3	0	0	1	2	1	1	0	0	0
4	2	2	1	2	3	0	0	0	0
5	0	0	0	0	1	0	0	0	0
6	3	1	1	1	3	0	0	0	0
7	0	3	3	0	1	0	0	0	0
8	1	0	1	2	4	0	0	0	0
9	0	1	0	0	0	0	0	0	0
10	0	2	0	0	0	0	0	0	0
Total colonies	6	9	8	8	13	1	0	0	0

been using routinely in the delivery and operating rooms. Merthiolate and mercresin occasionally produced skin irritation; this appeared as folliculitis.

Morbidity studies are of little or no value because of the relatively minor rôle played by the antiseptic in preventing morbidity. There was no demonstrable difference in obstetrics between this group and a comparable preceding group of cases.

No interference with wound healing was observed which could be attributed to phemerol.

We feel again that the antiseptic is a relatively minor rôle in skin antisepsis, but that it is involved in the process.

A variety of antiseptics have been published, but none relate directly to the skin. We believe that the antiseptic cannot be compared to the skin, and that the antiseptic is involved in the process.

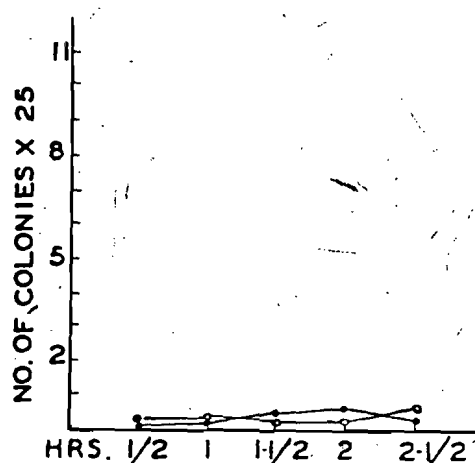
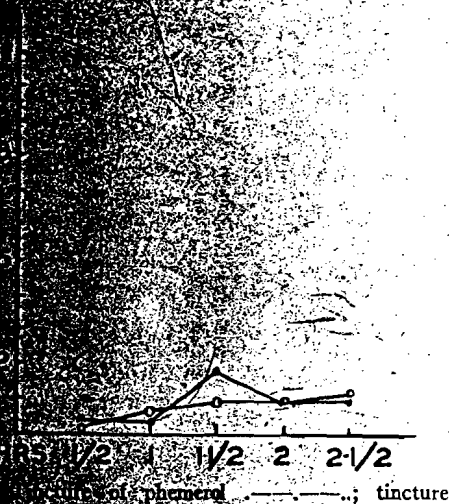


Fig. 6. Phemerol on heated thigh. —•—•—; phemerol on normal thigh o—o—o.

irritation and toxic effects; (2) be bacteriostatic and nonpathogenic organisms on the skin surface; (3) maintain its action throughout the duration of use; (4) not be affected by drying or by body fluids; (5) not interfere with healing. We have tried to study phemerol in relation to these criteria.

The limitations of this study are somewhat obvious. First, from the conditions of the study, conditions that do not agree with the clinical conditions of operating rooms. The temperature, humidity, soap and antiseptic used were not ideal. While this study showed a reduction of bacterial colonies, it did not show a reduction of the abdominal flora. Second, the use of clinical conditions in this study gives us a more realistic comparison. Second, we used the same technique on the same abdomen. This technique is a technique for the evaluation of skin antiseptics. It is not a technique for the comparison of antiseptics. The comparison of antiseptics with a varying bacterial flora is a technique for the evaluation of antiseptics.

The results of the bacteria identified in the study showed that most of the bacteria considered pathogenic are fairly resistant. Staphylococcus aureus and Streptococcus pyogenes were found on plates of all the antiseptics, indicating that under clinical con-

ditions as simulated in the study, these organisms are not consistently destroyed. In future studies of skin antiseptics more attention should be paid to the staphylococcus as they exist on the skin, rather than the easily killed streptococcus, pneumococcus, and bacilli of the typhoid-colon group. These latter organ-

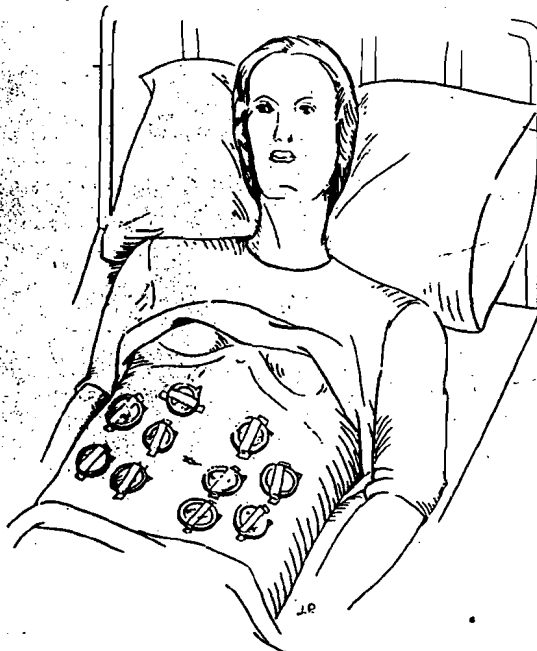


Fig. 7. Diagram of technique. The two sides of the abdomen were used for study; one side was treated with phemerol and the other side with the substance being compared. The plates were removed at one-half-hour intervals.

the antiseptics commonly employed at the present time.

It has been our hope to devise a technique by which we could study the penetration of antiseptics into the skin. Several unsuccessful attempts were made to precipitate antiseptic agents in the skin, thereby permitting microscopic identifications of the drug. However, the use of sweating gives us an approximate answer, for if the bacteria in the depths of the hair follicles and sweat glands were unaffected by phemerol, we would expect a rapid rise in the bacterial count as the plates were left on the heated thigh. Since this is not true (see Table VII and Fig. 6), it suggests that phemerol either penetrated the skin glands as a bactericide, or remained an active antiseptic when mixed with perspiration.

SUMMARY

A method of studying the various skin antiseptics commonly used in surgery has been presented. This technique employs small culture plates in direct contact with the abdomen, and uses the two sides of the abdomen for comparative data. The procedure simulates the conditions of clinical surgery, and we

of comparing clinical skin antiseptics.

A total of 1020 culture plates from 102 patients, containing over 50,000 bacterial colonies, have been studied.

Members of the following groups of antiseptics have been studied: detergents, tincture of soap and alcohol, cresol-mercurials, organic mercurials, and halogens.

When tested by this technique, tincture of phemerol of the detergent group was superior to the mercurials studied, and to tincture of green soap and alcohol; it seemed about equal to tincture of iodine.

CONCLUSION

By the technique employed in this study we find that tincture of phemerol (a) is a good bactericidal and bacteriostatic agent, (b) does not cause skin irritation, and (c) does not interfere with wound healing. It is an excellent skin antiseptic for use in surgery and obstetrics.

REFERENCES

1. BEATH, T. *Surgery*, 10: 4, 1930.
2. NOVAK, M., and HALL, H. *Surgery*, 10: 4, 1930.
3. SCOTT and HILL. *J. Am. Med. Ass.*, 1935, 61: 333.
4. VAICHULIS, J. A., and ARNOLD, J. A. *Surgery*, 1935, 61: 333.

Final Report on the Safety Assessment of Benzethonium Chloride and Methylbenzethonium Chloride

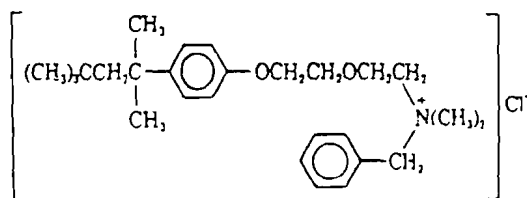
Benzethonium Chloride and Methylbenzethonium Chloride are quaternary ammonium salts used in cosmetics primarily as preservatives and secondarily as cationic surfactants, usually at concentrations below 1 percent. They can be irritating to the skin at concentrations of greater than 5 percent. Chronic and subchronic feeding studies indicated little or no toxic effects for both ingredients. Benzethonium Chloride was nonmutagenic in microbial systems and shown to be noncarcinogenic in rodent studies.

In clinical studies, Benzethonium Chloride produced mild skin irritation at 5 percent but not at lower concentration. Neither ingredient is considered to be a sensitizer.

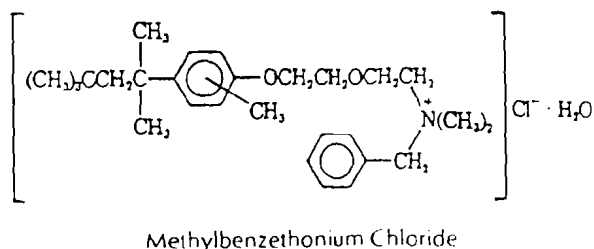
It is concluded that both compounds are safe at concentrations of 0.5 percent in cosmetics applied to the skin. A maximum concentration of 0.02 percent is safe for cosmetics used in the eye area.

CHEMICAL AND PHYSICAL PROPERTIES

Benzethonium Chloride, also known as diisobutyl phenoxy ethoxy ethyl dimethyl benzyl ammonium chloride and phemerol, and Methylbenzethonium Chloride, also known as diisobutyl cresoxy ethoxy ethyl dimethyl benzyl ammonium chloride, are synthetic quaternary ammonium salts.^(1,2) These two compounds conform to the following structural formulas:⁽³⁾



Benzethonium Chloride



Benzethonium Chloride and Methylbenzethonium Chloride occur as colorless to white, odorless to mildly odorous, and bitter tasting monohydrate crystals. Benzethonium Chloride, when crystallized from benzene, chloroform, or ether, forms thin hexagonal plates. Both compounds are soluble in water, the lower alcohols, glycols, ethyleneglycol monomethyl ether, tetrachloroethane, and benzene and miscible in ethylene dichloride and carbon tetrachloride. Benzethonium Chloride is additionally soluble in acetone and chloroform, and methylbenzethonium Chloride in cellosolve. Solutions of these compounds are stable within the pH range of 4.8 to 7.01.^(1,4-8) Ultraviolet (UV) spectra of Benzethonium Chloride and Methylbenzethonium Chloride indicate that these compounds have a peak absorbance at approximately 274 and 275 nm, respectively.^(9,10) The physicochemical properties of these compounds are presented in Table 1.

Quaternary ammonium compounds can be synthesized via multiple routes, beginning with fatty acids, fatty alcohols, or a hydrocarbon source. However, the exact methods of manufacture for Benzethonium Chloride and Methylbenzethonium Chloride are proprietary information. After developing the appropriate tertiary amine, Benzethonium Chloride can be prepared by heating the amine with the quaternizing agent benzyl chloride at 60 to 80°C in the presence of water. Manufacturers use a 5 to 25 percent excess of the stoichiometric quantity of tertiary amine to ensure complete consumption of the toxic benzyl chloride. Hydrochloric acid is then added to convert the unreacted tertiary amine to its hydrochloride salt. Formulations incorporating Benzethonium Chloride and Methylbenzethonium Chloride must therefore have an acid pH to prevent conversion back to the free amine. Methylbenzethonium Chloride is a methyl derivative of Benzethonium Chloride with the methyl group on the benzene ring containing the phenoxy group.^(7,8,14-16)

These commercial preparations contain a large number of functional groups and impurities including unreacted starting materials and side reaction products, such as amides and primary and secondary amines. Impurities from the quaternizing agent benzyl chloride may include traces of benzyl chloride itself, benzal chloride, benzyl alcohol, and benzaldehyde. Other impurities include benzylamine derivatives, sodium chloride, and hydrochloric acid. These impurities contribute greatly to the properties of the quaternary ammonium product. Gerstein⁽¹⁵⁾ even states: "The behavior of a quaternary material is not influenced, but rather determined, by the impurities and additives." No data are available on nitrosamine impurities.

Qualitative and quantitative determinations of Benzethonium Chloride and

TABLE 1. Physicochemical Properties of Benzethonium Chloride and Methylbenzethonium Chloride

Property	Value		Reference
	Benzethonium Chloride	Methylbenzethonium Chloride	
Empirical formula			
Anhydrous	$C_{17}H_{21}ClNO_2$	$C_{18}H_{23}ClNO_2$	3
Molecular weight			
Anhydrous	448.09	462.11	3, 11
Monohydrate	466.11	480.13	
Melting point (°C)	158-166 (sinters at 120)	159-163	1, 3, 4, 6
Density (lb/ft ³)	27.5	27.5	7, 8
Assay as ingredient (anhydrous basis)	97.0-103.0%	97.0-103.0%	3
Loss on drying (105° for 4 hours)	≤ 5%	≤ 5%	3, 12
Residue on ignition	≤ 0.1%	≤ 0.1%	3
Solubility*			
Water	s. vs	s. vs	4, 6-8, 13
Alcohol	s	s. vs	4, 6-8, 13
Acetone	s	-	4, 6
Benzene	s	vs (hot)	1, 4, 6
Carbon tetrachloride	m	m	4, 7, 8
Cellosolve	-	s. vs	4, 6, 13
Chloroform	s	s. vs	4, 6, 13
Ether	ss, s	s, s. vs	4, 7, 8, 13
Ethylene dichloride	m	m	7, 8
Glycols	s	s	7, 8
Tetrachloroethane	s	s	7, 8
pH			
10% solution	7.01	-	5
1% solution	4.8 to slightly alkaline	Neutral to slightly alkaline	6, 13
Surface tension (10% solution)	36 dynes/cm	-	5
Wetting power (0.1% solution)	142 seconds	-	5
Foam height (1.0% solution)	268 nm	-	5
UV spectra-peak absorbance (nm)	274	275	9, 10

*i, insoluble; m, miscible; s, soluble; ss, slightly soluble; vs, very soluble

Methylbenzethonium Chloride have been made by many methods, including colorimetry, chromatography, multiphase titrations, extraction and spectrophotometry, electrophoresis, ultraviolet spectroscopy, potentiometric assays, and various other physical and chemical assays. Many of these depend on the formation of a relatively stable ion-pair complex. Tanaka et al.⁽¹⁷⁾ report that the extractability of quaternary ammonium compounds as an ion-pair complex with bromophenol blue is determined by the lipophilic character of the ion and the steric effect around the cationic head. Table 2 lists the reported analytical methods for Benzethonium Chloride and Methylbenzethonium Chloride determination.

The reactivity of Benzethonium Chloride and Methylbenzethonium Chloride is determined for the most part by their cationic properties. They are inactivated by and are incompatible with soaps, anionics, organic material, nitrates, iodides, hexachlorophene, potassium chromate, potassium dichromate, sodium hepta-

TABLE 2. Analytical Methods for the Determination of Benzethonium Chloride and Methylbenzethonium Chloride

Method	Reagents and Specifics	Interference	Reference
Agar gel electrophoresis			24
Colorimetric	Bromophenol blue, sodium hydroxide		13, 17, 25
	Sodium alizarine sulfonate		26
	Eosin		27
Colorimetric/ion exchange/gas-liquid chromatography (GLC)	Bromophenol blue, sodium hydroxide		25
Electrophoresis	Iron or aluminum anodes		28
Extraction/spectrophotometric	2,6-dibromophenol indophenol, 1,2-dichloroethane, maximum absorption of extraction at 640 nm	None at pH 5.6; other quaternary ammonium salts and amines at pH 8.2	29
	2,6-dichloroindophenol, nitrobenzene, maximum absorption of extraction at 650 nm	None from common inorganic salts; slight from amines and alkaloids	30
Gas chromatography (GC); mass spectrometry			31
Ion-pair atomic absorption	Sodium dioctylsulfosuccinate, cupric orthophenanthroline, methyl isobutyl ketone		32
Multiphase titrations	BC: Water, chloroform, sodium tetraphenylborate, bromophenol blue, sodium hydroxide, water, hydrochloric acid, (HCl), sodium tetraphenylboron, methyl orange MBC: Water, chloroform, potassium iodide, potassium iodate, HCl		12, 13, 33
Potentiometric assay	Mercury-coated platinum, potassium ion selective, or silver electrodes, sodium tetraphenylborate	None from alcohol, acetone, sodium phosphates, disodium edetate, nonionic surfactants	34-37
Reverse phase ion-pair chromatography	Perchloric acid, methane-sulfonic acid		38
Spectrophotometric assay	Bromthymol blue at pH 7.5	None from epinephrine bitartrate, phenylephrine-HCl, pilocarpine-HCl, polyvinyl alcohol	39
Thin-layer chromatography (TLC)			40
TLC/fluorescence/refractive index			41
Ultraviolet spectroscopy	BC: Maximum absorption at 256-275 nm		12, 27
Various other physical and chemical assays			12, 13

phosphate, cotton fabrics, cellulose sponges, certain plastics, and other porous materials. Benzethonium Chloride (in greater than 2 percent concentrations) is precipitated from mineral acids and salt solutions as an oil that recrystallizes on drying. Both compounds are considered biologically active due to their precipitation, denaturation, redispersion, and complex formation reactions with proteins. Benzethonium Chloride and Methylbenzethonium Chloride are readily adsorbed onto a variety of surfaces, including proteinaceous surfaces, gauze, cork, plastics, and cellulose. As antimicrobial agents they adsorb onto the negatively charged cell wall of microorganisms, interrupt normal cell metabolism, and lead to death or growth inhibition.^(1, 4, 6-8, 15, 18-23)

Benzethonium Chloride and Methylbenzethonium Chloride are relatively stable compounds; both aqueous and alcoholic solutions are stable in light, air, and temperatures up to 100°C. They are stabilized in detergents, cosmetics, and pharmaceuticals containing oxygen-forming substances by the presence of organotin compounds. Gamma-irradiation was found to decrease the antibacterial activity of Benzethonium Chloride, although this effect became less severe as the concentration of Benzethonium Chloride increased. Irradiation also increased the surface tension of 0.01 to 0.1 percent solutions; however, little effect was noted on 1 percent solutions.^(1, 22, 42, 43)

In a study on sarcosinate-cationic creme rinse shampoos, Benzethonium Chloride was compatible with many sarcosinate surfactants while retaining its antimicrobial activity.⁽⁴⁴⁾ Methylbenzethonium Chloride had an increase in bacteriostatic action when combined with sodium lauroyl sarcosinate.⁽⁴⁵⁾ These quaternary compounds also molecularly bind other formulation ingredients to the surface of the hair, thus intensifying the effects of fatty alcohols and esters, perfume oils, and other waxy and oily compounds.⁽¹⁵⁾

Benzethonium Chloride loses its antimicrobial activity when formulated with such cosmetic ingredients as lecithin (0.3 percent), polysorbate 80 (1.0 percent), and sodium sulfite (0.1 percent).⁽⁴⁶⁾ Methylbenzethonium Chloride in a 0.1 percent aqueous solution was inactivated by adsorption onto a variety of powders, including calamine, heavy and light kaolin, and magnesium trisilicate after 18 hours of storage at 22°C. Autoclaving Methylbenzethonium Chloride with the powders for 15 minutes at 121°C decreased the amount of Methylbenzethonium Chloride adsorbed.⁽⁴⁷⁾ Germicidal activity of both quaternary ammonium compounds was decreased by metallic ions in cosmetic formulations. This decrease was proportional to the valence of the ion, which interfered by competing for the negative sites on the microbial cell wall.⁽²⁷⁾

USE

Cosmetic Use

Benzethonium Chloride and Methylbenzethonium Chloride are used in cosmetics as preservatives, antimicrobials, and cationic surfactants. They are usually found at concentrations below 1 percent in the following product categories: baby, bath, eye makeup, personal cleanliness, fragrance, noncoloring hair, shaving, skin, and suntan preparations.^(15, 44, 48)

The FDA product formulation data for Benzethonium Chloride and Methylbenzethonium Chloride are compiled through voluntary filing of such data in accordance with Title 21 part 720.4 of the Code of Federal Regulations⁽⁴⁹⁾ (Table 3). Ingredients are listed in prescribed concentration ranges under specific product type categories.⁽⁴⁸⁾ Since certain cosmetic ingredients are supplied by the manufacturer at less than 100 percent concentration, the value reported by the cosmetic formulator may not necessarily reflect the actual concentration found in the finished product; the actual concentration in such a case would be a fraction of that reported to the FDA. The fact that data are only submitted within the

TABLE 3. Product Formulation Data⁽⁴⁸⁾

Product Category*	Total No. of Formulations in Category	Total No. Containing Ingredient	No. of Product Formulations Within Each Concentration Range (%)		
			> 1-5	> 0.1-1	≤ 0.1
Benzethonium Chloride					
Baby products	15	1	-	1	-
Bath preparations	132	1	-	-	1
Eyeliners	396	2	-	2	-
Colognes and toilet waters	1120	6	-	6	-
Perfumes	657	3	-	3	-
Fragrance powders (dusting and talcum excluding aftershave talc)	483	3	-	3	-
Hair conditioners	478	2	-	2	-
Hair sprays (aerosol fixatives)	265	1	-	-	1
Hair rinses (noncoloring)	158	3	-	-	3
Hair shampoos (noncoloring)	909	1	-	1	-
Tonics, dressings, and other hair grooming aids	290	1	-	-	1
Wave sets	180	1	-	-	1
Other hair preparations (noncoloring)	177	2	-	-	2
Deodorants (underarm)	239	11	-	8	3
Douches	26	7	4	3	-
Feminine hygiene deodorants	21	3	-	-	3
Other personal cleanliness products	227	7	-	3	4
Aftershave lotions	282	2	-	-	2
Men's talcum	13	2	-	1	1
Preshave lotions (all types)	29	1	-	1	-
Skin cleansing preparations (cold creams, lotions, liquids, and pads)	680	5	-	1	4
Face, body, and hand skin care preparations (excluding shaving preparations)	832	7	-	2	5
Moisturizing skin care preparations	747	2	-	-	2
Paste masks (mud packs)	171	2	-	-	2
Skin fresheners	260	12	-	1	11
Other skin care preparations	349	3	-	-	3
Suntan gels, creams, and liquids	1642	2	-	-	2
Indoor tanning preparations	15	3	-	-	3
1981 TOTALS		93	4	36	53

TABLE 3. (Continued)

Product Category*	Total No. of formulations in Category	Total No Containing Ingredient	No. of Product Formulations Within Each Concentration Range (%)*		
			> 1-5	> 0.1-1	≤ 0.1
<i>Methylbenzethonium Chloride</i>					
Baby lotions, oils, powders, and creams	56	2	-	1	1
Colognes and toilet waters	1120	1	-	-	1
Hair conditioners	478	1	-	-	1
Hair sprays (aerosol fixatives)	265	6	-	-	6
Deodorants (underarm)	239	5	-	4	1
Douches	26	1	-	1	-
Feminine hygiene deodorants	21	2	-	-	2
Other personal cleanliness products	227	1	-	-	1
Aftershave lotions	282	4	-	3	1
Other shaving preparation products	29	1	-	-	1
Skin cleansing preparations (cold creams, lotions, liquids, and pads)	680	1	-	-	1
Face, body, and hand skin care preparations (excluding shaving preparations)	832	1	-	-	1
Moisturizing skin care preparations	747	1	-	-	1
Skin fresheners	260	3	-	-	3
Suntan gels, creams, and liquids	164	2	-	-	2
Other suntan preparations	28	1	-	-	1
1981 TOTALS		33	0	9	24

*Preset product categories and concentration ranges in accordance with federal filing regulation (21 CFR 720.4)

framework of preset concentration ranges also provides the opportunity for over-estimation of the actual concentration of an ingredient in a particular product. An entry at the lowest end of a concentration range is considered the same as one entered at the highest end of that range, thus introducing the possibility of a 2- to 10-fold error in the assumed ingredient concentration.

In 1981, approximately 96 and 97 percent of the formulations containing Benzethonium Chloride and Methylbenzethonium Chloride, respectively, incorporated these ingredients at concentrations of 1 percent or less. Furthermore, 57 and 71 percent of the total 93 Benzethonium Chloride and 34 Methylbenzethonium Chloride formulations, respectively, contained concentrations of 0.1 percent or less.⁽⁴⁸⁾

The European Economic Community (EEC) has approved a maximum concentration of 0.1 percent for Benzethonium Chloride as a provisionally permitted preservative in cosmetics.⁽⁴⁹⁾ No further limitations or label requirements of any kind are listed.

The formulation data presented in Table 3 indicate that cosmetic products containing Benzethonium Chloride and Methylbenzethonium Chloride may

contact all external body surfaces and hair, as well as the eyes and mucosal membranes. These products may be used daily or occasionally over a period of up to several years. The frequency and length of application could result in continuous exposure.

Noncosmetic Use

Benzethonium Chloride and Methylbenzethonium Chloride are widely used in disinfectants, germicides, herbicides, bactericides, topical anti-infectives, as cationic detergents, and preservatives.^(4,6,14-16,20,27,51-60) Their varied applications as disinfectants encompass use in restaurants (on eating equipment), dairy farms (on milking equipment), janitorial purposes, food plants, hospitals, barber and beauty shops, textile factories, food storage rooms, and swimming pools. Additionally, the veterinary and agricultural uses of Benzethonium Chloride include sanitizing chicken drinking water, in egg handling, as general disinfectants, and as topical antibacterials. As cationic surfactants, these compounds are used in ore flotation, fabric softening, colloid flocculation processes, asphalt emulsification, corrosion inhibition, paper processing wood pulp slurries, and as pigment wetting and grinding aids in the production of thixotropic paints and printing inks.

Benzethonium Chloride ~~is~~ has been used in various pharmaceuticals primarily as a preservative. Benzethonium Chloride has been incorporated into vaccines, preparations for treating cardiovascular disorders, anesthetics, and injectable solutions and is also used in the production of heparin derivatives. Benzethonium Chloride concentrations vary from product to product but seldom exceed 1 percent.^(27,51-67) In addition, 0.2 percent aqueous or alcoholic solutions of Benzethonium Chloride have been used in the treatment of hydrofluoric acid burns.^(68,69)

Benzethonium Chloride and Methylbenzethonium Chloride are currently under evaluation by the FDA Over-the-Counter (OTC) Drug Review Program. These compounds were assigned to 8 of the 17 advisory panels pertaining to their use as antimicrobials, in contraceptives and other vaginal products, dentifrices and dental care agents, miscellaneous external, ophthalmic, and oral cavity drug products, topical analgesics, antirheumatics, otic, burn, and sunburn treatment and prevention products.⁽⁷⁰⁾ The Ophthalmic Panel found Benzethonium Chloride satisfactory as a preservative at maximum concentrations of 0.01 percent for preparations used directly in the eye and at a maximum of 0.02 percent for preparations not for direct use in the eye.⁽⁷¹⁾ The Antimicrobial I Panel also concluded that Benzethonium Chloride and Methylbenzethonium Chloride are safe and effective for use as detergents in skin wound cleansers at a maximum concentration of 0.13 percent; no claims of antimicrobial activity are associated with this use.⁽⁷²⁾ Several panels noted that although quaternary ammonium compounds were embraced as disinfectants upon their appearance in 1935, subsequent reviews have produced significant doubt as to their safety and antimicrobial effectiveness.^(21,71-73) Controversy surrounds their microbial spectrum (particularly gram-negative bacteria) and inactivation by a substantial number of compounds. Consequently, the major classification of Benzethonium Chloride and Methylbenzethonium Chloride as used in OTC products falls in Category III, insufficient data available for final evaluation of safety and effectiveness. Benzethonium Chloride and Methylbenzethonium Chloride have been classified as

inactive ingredients in contraceptives: Benzethonium Chloride in dentifrices, ophthalmic solutions, and hair growth and hair loss prevention products, and Methylbenzethonium Chloride in acne treatment products.^(21,70,71,74,75) Table 4 presents a synopsis of the status of Benzethonium Chloride and Methylbenzethonium Chloride in the OTC drug review.

Benzethonium Chloride and Methylbenzethonium Chloride are included in the listing of quaternary ammonium chlorides (hexadecyl, octadecyl derivative) as indirect food additives, limited to use as preservatives only in adhesives used in packaging, transporting, or holding food.⁽⁷⁶⁾ The literature also contains references to the use of Benzethonium Chloride in India as a plant growth regulator applied to sugar cane foliage. Five to six weeks treatment with 1000 and 2500 ppm Benzethonium Chloride improved the sugar cane purity coefficient and increased the percentage of fiber.^(77,78)

Benzethonium Chloride and Methylbenzethonium Chloride are also used in several analytical methods: an aqueous Zimmermann reaction test for the determination of 17-ketosteroids (Benzethonium Chloride), a microturbidimetric method for the determination of protein in cerebrospinal fluid and urine (Benzethonium Chloride), and in combination with toluene and octoxynol-9 in a scintillator for colloidal counting of plasma and urine (Methylbenzethonium Chloride).⁽⁸³⁻⁸⁵⁾

GENERAL BIOLOGY

Antimicrobial

The antimicrobial properties of Benzethonium Chloride and Methylbenzethonium Chloride have been extensively studied. Their lack of odor, color, instability, and toxicity (at effective levels) has resulted in their widespread use as disinfectants and preservatives since 1935.^(22,73) However, varying experimental and in-use results have engendered controversy over the scope of their microbial spectrum and their inactivation by a large number of materials. This has led the majority of FDA OTC Drug Review Panels to conclude that insufficient data are available to determine the safety and efficacy of Benzethonium Chloride and Methylbenzethonium Chloride as used in OTC drug products (Table 4).

Benzethonium Chloride and Methylbenzethonium Chloride are inactivated by soaps, anionics, phospholipids, proteins, nitrates, iodides, polysorbate 80, sodium sulfite, magnesium, calcium, and iron salts.^(1,6,20,22,46) Hard water, acidity, and the presence of organic matter also generally reduce antimicrobial effectiveness. The surface-active nature of these compounds, causing them to be readily adsorbed on glass or plastic surfaces, also accounts for some reduction in effectiveness.^(20-22,73)

The germicidal action of Benzethonium Chloride and Methylbenzethonium Chloride has generally been credited to their ability to disrupt cell membrane permeability and the subsequent loss of intracellular materials. Many other factors are believed to add to the sum total mechanism, varying in relative influence with changing conditions. These include lysis, protein denaturation, oxidation and enzyme inhibition, effects on activating ions, and interference with growth and reproduction.^(23,73,86-89)

TABLE 4. Status of Benzethonium Chloride and Methylbenzethonium Chloride in the OTC Drug Review^{1,2,3}

<i>Ingredient</i>	<i>Advisory Review Panel</i>	<i>Active (A) or Inactive (II)*</i>	<i>Use/Comment</i>	<i>Recommended Category†</i>	<i>Reference Document‡</i>	<i>Date</i>
Benzethonium Chloride and Methylbenzethonium Chloride	Antimicrobial I	A	Antimicrobial soap/physical and/or chemical incompatibility in formulation	II SE	TFM (73)	1/6/78
Benzethonium Chloride and Methylbenzethonium Chloride	Antimicrobial I	A	Surgical hand scrub	III SE	TFM (73)	1/6/78
Benzethonium Chloride and Methylbenzethonium Chloride	Antimicrobial I	A	Skin wound cleanser (maximum concentration of 1/750)	I	TFM (pre-amble Proposal (72)	9/13/74
Benzethonium Chloride and Methylbenzethonium Chloride	Antimicrobial I	A	Skin antiseptic	III E	TFM (73)	1/6/78
Benzethonium Chloride and Methylbenzethonium Chloride	Antimicrobial I	A	Health care personnel handwash	III SE	TFM (73)	1/6/78
Benzethonium Chloride and Methylbenzethonium Chloride	Antimicrobial I	A	Skin and protectant	III E	TFM (73)	1/6/78
Benzethonium Chloride and Methylbenzethonium Chloride	Antimicrobial I	A	Patient preoperative skin preparation	III SE	TFM (73)	1/6/78
Benzethonium Chloride and Methylbenzethonium Chloride	Contraceptives and other vaginal drug products	I	Caution: surfactant in douches (BC) and contraceptives (MBC), preservative in vaginal preparations	III SE	Proposals (21)	12/12/80
Benzethonium Chloride	Antimicrobial II	A	Antifungal	III SE	OTC Panel Mtg. (28th)	8/26-27/77

Benzethonium Chloride	Dentifrices and dental care agents	I	Ingredient contained in marketed products submitted for review, considered inactive	-	Proposal (74)	3/28/80
Benzethonium Chloride	Miscellaneous external drug products	A	Styptic	II	OTC Panel Mtg. (40th)	8/3-4/80
Benzethonium Chloride	Miscellaneous external drug products	A	Antimicrobial (any concentration) as an aid in prevention of diaper rash, cradle cap, excoriating skin conditions, and to stimulate healing	III E	OTC Panel Mtg. (41st)	10/5-6/80
Benzethonium Chloride	Miscellaneous external drug products	I	Hair grower and hair loss prevention products	-	Proposal (75)	11/7/80
Benzethonium Chloride	Ophthalmic drug products	I, A	Preservative agent in maximum concentrations of 0.01% in the eye considered inactive only when used as a formulation agent and when no labeling claims are made	-	Proposals (75)	5/6/80
Benzethonium Chloride	Oral cavity drug products	A	Antimicrobial used for oral health care	III SE	OTC Panel Mtgs. (27th, 28th)	8/14/79, 12/12-14/79
Benzethonium Chloride	Topical analgesics, antirheumatics, otic, burn, sunburn treatment and prevention products		Deferred to Antimicrobial Panel	-	Proposal (80)	12/4/79
Benzethonium Chloride (In Karaya/Tragacanth as a vehicle)	Miscellaneous external drug products	A	Antiinfective. Discussion delayed until receipt of further information	-	OTC Panel Mtg. (21st)	9/30-10/1/77
Benzethonium Chloride and Captan	Miscellaneous external drug products	A	Panel undecided on the effect of combined active ingredients as an antidandruff treatment	-	OTC Panel Mtg. (38th)	4/20-21/80

TABLE 4. (Continued)

<i>Ingredient</i>	<i>Advisory Review Panel</i>	<i>Active (A) or Inactive (I)*</i>	<i>Use/Comment</i>	<i>Recommended Category†</i>	<i>Reference Document‡</i>	<i>Date</i>
Methylbenzethonium Chloride	Antimicrobial II	I	Inactive ingredient in treatment of acne	—	OTC Panel Mtg. (43rd)	7/20/79
Methylbenzethonium Chloride	Miscellaneous external drug products	A	Treatment of cradle cap (seborrheic dermatitis of the scalp in infants)	III S	OTC Panel Mtg. (40th)	8/3-4/80
Methylbenzethonium Chloride	Miscellaneous external drug products	A	Corn and callus remover	II SE	Proposal (FDA, 1982)	1/5/82
Quaternary ammonium compounds (includes Benzethonium Chloride and Methylbenzethonium Chloride)	Antimicrobial I	A	Use concentration not greater than 0.13%	I	TFM (FDA, 1978)	1/6/78
Quaternary ammonium compounds (includes Benzethonium Chloride and Methylbenzethonium Chloride)	Contraceptives and other vaginal products		BC considered safe and effective in a vaginal douche in recommended dosage dilutions and with directions for intermittent usage, no claims of anti-septic or disinfectant activity	—	OTC Panel Mtg. (19th)	6/23-24/75
Quaternary ammonium compounds (includes Benzethonium Chloride and Methylbenzethonium Chloride)			MBC considered effective but of unproven safety as cleaning and deodorizing components of vaginal suppositories, unreliable as bactericides	—	OTC Panel Mtg. (19th) OTC Panel Mtg. (30th)	12/16-17/76

Quaternary ammonium compounds (includes Benzethonium Chloride and Methylbenzethonium Chloride)	Ophthalmic drug products	A	Incompatible with serum. Serum and/or yeast cells may inactivate preservative system	-	OTC Panel Mtg. (24th)	2/3-4/78
Quaternary ammonium compounds (includes Benzethonium Chloride and Methylbenzethonium Chloride)	Oral cavity drug products	A	Antimicrobial for use on oral and pharyngeal mucous membranes	III SE	OTC Panel Mtg. (27th)	8/14/79

*Active ingredient: "any component that is intended to furnish pharmacological activity or other direct effect in the diagnosis, cure, mitigation, treatment, or prevention of disease, or to affect the structure or any function of the body of man or other animals. The term includes those components that may undergo chemical change in the manufacture of the drug product and be present in the drug product in a modified form intended to furnish the specified activity or effect." (1) Inactive ingredient, "any component other than an 'active ingredient.'" (1)(2)

†Category I. Conditions under which OTC drug products are generally recognized as safe and effective and are not misbranded.

Category II. Conditions under which OTC drug products are not generally recognized as safe and effective or are misbranded.

Category III. Conditions for which the available data are insufficient to permit final classification at this time as Category I or II.

Categories II and III: reason for categorization may be symbolized by S (safety) and/or E (effectiveness).

- Indicates no categorization at this time.

*TFM, Tentative final monograph.

Benzethonium Chloride and Methylbenzethonium Chloride are effective against many gram-positive and some gram-negative bacteria, the former generally being more susceptible to their germicidal action, whereas many strains of the latter, particularly *Pseudomonas aeruginosa*, are resistant.^(1,22,23,73,90-92) Christensen⁽⁹³⁾ obtained satisfactory bactericidal activity (at 37°C) with concentrations of Benzethonium Chloride ranging from 0.005 to 0.01 percent; concentrations of 0.0025 percent Benzethonium Chloride, intended for use in vaccines, had insufficient antibacterial effects. Rawlins et al.⁽⁹⁴⁾ and Joslyn et al.,⁽¹⁾ on the other hand, have reported satisfactory activity (at 20°C) with Benzethonium Chloride concentrations of 0.00125 to 0.0083 percent.

Benzethonium Chloride and Methylbenzethonium Chloride have less fungicidal than bactericidal activity.⁽⁷³⁾ Concentrations of Benzethonium Chloride ranging from 0.1 to 0.2 percent were effective against some fungi and ineffective against others.^(1,94)

Benzethonium Chloride has synergistic antibacterial and antifungal activity with acylated peptides,⁽⁹⁵⁾ chlorhexidine gluconate,⁽⁹⁶⁾ candidin, a polyene macrotide antifungal antibiotic,⁽⁹⁷⁾ and thimerosal.⁽⁹³⁾

Benzethonium Chloride has been tested widely for its potential to reduce bacterial plaque accumulation. Results of numerous studies indicate that solutions or mouthrinses containing Benzethonium Chloride (0.075 to 0.1 percent) give a moderate to significant reduction in plaque accumulation.⁽⁹⁸⁻¹⁰²⁾ One clinical study found no significant reduction in existing plaque accumulations.⁽¹⁰³⁾ Benzethonium Chloride was slightly less effective in inhibiting plaque formation than chlorhexidine and chlorhexidine gluconate, whereas zinc chloride reduced the antiplaque potency of this compound.^(99,104,105) Several investigators have reported yellow-brown tooth and tongue discolorations associated with the use of Benzethonium Chloride-containing dentifrices.^(99,106,107) Gaffar and Volpe^(108,109) reported that the dental staining can be prevented by the incorporation of a polyamine polyphosphonate without inactivating Benzethonium Chloride.

A process has been developed for incorporating Corobex CP-4 containing 0.075 percent Benzethonium Chloride into polymerized methyl methacrylate used in contact lenses. The treated lenses had reduced numbers of organisms, and thus treatment would lessen bacterial and other contamination of ocular tissues.⁽¹¹⁰⁾

Benzethonium Chloride has also been used for many years as a bacterial inactivator in the manufacture of vaccines. Marked losses in vaccine potency, first reported in 1960, necessitated numerous studies on the antimicrobial effectiveness of Benzethonium Chloride in the poliomyelitis, pertussis, diphtheria-pertussis-tetanus (DPT), and the combined DPT-poliomyelitis (DPTP) vaccines.^(119,27,61,111,112) Pivnick et al.⁽⁶¹⁾ found Benzethonium Chloride, at 25 ppm, to be an ineffective inhibitor of gram-negative bacteria in both the poliomyelitis and DPTP vaccines. However, Benzethonium Chloride did inhibit growth of gram-positive bacteria, yeasts, and mold while also increasing the antifungal activity of other preservatives in the vaccines.

Biochemical Effects

Benzethonium Chloride inhibited proteolytic enzymes, including brain aminopeptidases and arylamidases, trypsin (Methylbenzethonium Chloride in-

hibits also), and bovine hypothalamus acid proteinase.^(86,113-117) Stedman et al., in their studies on *Serratia marcescens*, emphasized the contributing role this enzyme activity loss plays in the cytotoxic effects of Benzethonium Chloride. They found that approximately 50 percent enzyme inhibition was achieved with a Benzethonium Chloride concentration that gave less than a 2 percent loss in cell viability. Makinen⁽¹¹⁸⁾ found that Benzethonium Chloride markedly inhibited the enzymatic hydrolysis of all but 1 tested amino acid, 2-naphthylamides purified from human saliva. Kinetic data indicated that the inhibition was competitive and effective under high substrate conditions.

Sugiura and Ogiso⁽¹¹⁹⁾ studied the effect of Benzethonium Chloride on the enzymatic hydrolysis of olive oil by *Mucor* lipase. Benzethonium Chloride at a maximum concentration of 0.0065 percent enhanced the rate of hydrolysis. The investigators found that the increased rate depended on the oil:surfactant ratio and that a small amount of Benzethonium Chloride increased lipase adsorption at the oil-water interface. However, Benzethonium Chloride concentrations greater than 0.0065 percent resulted in lipase inhibition.

Several studies have been conducted on the effects of Benzethonium Chloride on acetylcholinesterase. Addition of Benzethonium Chloride to electric eel spinal cord or tissue preparations decreased the proportion of solubilized enzyme as globular species and slowed or inhibited the conversion of "native" species into globular forms.^(113,120) Benzethonium Chloride also had an inhibitory effect on acetylcholinesterase in homogenates from rabbit or ox caudate nuclei. The investigators suggested that inhibition was achieved through the complexing of the benzethonium cation with the anionic enzyme site.⁽¹²¹⁾

Several other biochemical effects of Benzethonium Chloride have been studied. Benzethonium Chloride (0.02 M) in a phosphate-buffered solution did not inhibit the photodecomposition of flavin adenine dinucleotide (FAD) after irradiation for 2 hours.⁽¹²²⁾ Benzethonium Chloride also induced UV spectral changes in drugs, such as thiamylal and thiopental, and enhanced ampicillin partition behavior.^(123,124)

Cellular Effects

Benzethonium Chloride and Methylbenzethonium Chloride are known to cause cytolytic injury by disrupting the permeability properties of cellular membranes with a subsequent loss of intracellular materials. Absolute cell density and the weight ratio of preservative to cells dictates the amount of lysis obtained. Significant reduction in either the cell density or in the weight ratio also reduces the amount of lysis. Other cytotoxic effects include protein denaturation, oxidation and enzyme inhibition, effects on activating ions, and interference with growth and reproduction.^(23,73,86-89,97,125,126)

In one of the many studies on Benzethonium Chloride, the compound was toxic at a concentration of 10 $\mu\text{g/ml}$ to 3 types of cultured human cells, whereas, 1 $\mu\text{g/ml}$ inhibited cell growth.⁽¹²⁷⁾ In another test for adjuvant activity using diphtheria toxoid in guinea pigs, Benzethonium Chloride was an active adjuvant, hemolytic, and disruptive to cellular cytoplasm.⁽¹²⁶⁾

In an ultrastructural study, suspensions of ram spermatozoa and avian erythrocytes were coincubated (45 minutes at 22°C) in the presence of Benzethonium

Chloride and Methylbenzethonium Chloride at concentrations ranging from 0 to 177 $\mu\text{g/ml}$. Erythrocyte swelling was induced as a result of increased membrane permeability. This, in turn, created a situation of close proximity with localized regions of membrane fusion and, in some cases (all concentrations of Benzethonium Chloride, only the highest concentration of Methylbenzethonium Chloride), erythrocyte-erythrocyte fusion. Spermatozoa with intact acrosomes were also observed embedded in erythrocyte cytoplasm; adjacent membranous vesicles were believed to represent the fused cellular membranes. Benzethonium Chloride and Methylbenzethonium Chloride have been used to accelerate the acrosome reaction in guinea pig spermatozoa and to produce acrosomal vesiculation in bovine sperm.⁽¹²⁵⁾

Benzethonium Chloride and Methylbenzethonium Chloride are also potent spermicides. A foam containing 0.2 percent Benzethonium Chloride was an effective spermicide.⁽¹²⁸⁾ Brotherton⁽¹²⁹⁾ tested Benzethonium Chloride and Methylbenzethonium Chloride for spermicidal activity by titration against human spermatozoa and found both effective. She also found that slight "variations" in chemical structure resulted in large potency differences: Methylbenzethonium Chloride, with an extra ring methyl group, was 3 times more potent than Benzethonium Chloride (1.82 pmol/cell Methylbenzethonium Chloride compared to 6.03 pmol/cell Benzethonium Chloride necessary for 100 percent stripping of spermatozoa).

Fur depigmentation has been noted in a number of studies on Benzethonium Chloride. In a study in which Benzethonium Chloride was injected subcutaneously into 50 black mice after an injection of dibenzo(a,i)pyrene (DBP), all of the mice receiving 2 injections of 0.7 mg Benzethonium Chloride at Days 1 and 8 had decolorization of the fur at the site of injection. Of those 50 receiving 0.35 mg Benzethonium Chloride on Days 1, 8, and 15, 97.4 percent had depigmentation. In another study, spotty depigmentation occurred 10 days after treatment in 2 of 3 mice painted with 140 mg/kg Benzethonium Chloride in tricaprylin. Other mice in this study received doses ranging from 8.75 to 280 mg/kg and did not have depigmentation. Hair exposed *in vitro* for 48 hours was not bleached by concentrations of Benzethonium Chloride up to 280 mg/kg. One hundred mice injected subcutaneously in the groin with 0.7 mg Benzethonium Chloride (in tricaprylin) also had fur depigmentation. In another study, all of the 8 mice receiving repeated subcutaneous injections of 70 mg/kg Benzethonium Chloride had depigmentation near the injection site at 34 days; 5 of the 8 mice receiving 35 mg/kg had a similar spotty depigmentation. When the depigmented fur was plucked, the new hair growth was also depigmented.⁽¹³⁰⁾

Tissue Effects

Several studies have been conducted on the tissue effects of Benzethonium Chloride. The ciliary activity of isolated mouse tracheal mucosa was weakened after a 15-minute contact with a Benzethonium Chloride concentration of 0.01 percent in Locke-Ringer's solution.⁽¹³¹⁾ A final Benzethonium Chloride dilution of 1:40,000 in Parker's medium 199 was toxic to tissue cultures of primary monkey kidney cells. The toxic action was also found to be time dependent.⁽¹³²⁾

The apparent exsorption rate constant K (excretion into the intestinal lumen through the intestinal wall) of sulfguanidine administered intravenously to rats

was used to measure the influence of Benzethonium Chloride upon the intestinal mucosa. The perfusion of Benzethonium Chloride in an isotonic (pH 7.4) phosphate buffer through the intestine at a rate of 4 ml/minute for 10 minutes greatly increased K and resulted in histological changes in the intestinal mucosa. The diffusion of sulfaguanidine from the blood vessel to the intestinal lumen was increased. The K values for Benzethonium Chloride were independent of the concentration (5 mM and 10 mM), and the investigators believed this was due either to good tissue permeability or a rate determining effect of blood or lymph flow.⁽¹³³⁾

The effects of Benzethonium Chloride on tone and motility of isolated segments of rabbit and rat ileum were studied by the Magnus technique. Benzethonium Chloride was added to the muscle preparation to give effective concentrations ranging from 0.00005 to 0.5 percent; fresh segments were used for each test concentration. Benzethonium Chloride inhibited the motility of the smooth muscle of the rabbit ileum. Concentrations of 0.002 to 0.5 percent totally inhibited muscle contractions and markedly decreased muscle tone. Some motility remained at 0.001 percent concentration, but muscle tone was equally depressed. Decreasing concentrations produced decreasing effects. At 0.0002 percent concentrations, tone was still depressed, but motility was only slightly affected; 0.0001 percent had only a slight effect on tone and no effect on motility. A concentration of 0.00005 percent produced no effects. The effects of Benzethonium Chloride on rat ileum were similar in their progression, but the results were indicative of a slightly greater sensitivity of the rat ileum. The investigators suggested that Benzethonium Chloride had a specific toxic action and reported that the inhibitory effect at concentrations as high as 0.005 percent could be reversed by several changes of Locke-Ringer's solution.⁽⁶⁰⁾

Absorption, Metabolism, and Excretion

Labeled ¹⁴C-Benzethonium Chloride at doses of 1.13 and 3.56 mg/kg per day was administered orally to pregnant rats on Days 6 through 15 of gestation. The fetal absorption of ¹⁴C-Benzethonium Chloride was variable; and most of the radioactivity remained within the dam.⁽¹³⁴⁾

ANIMAL TOXICOLOGY

Acute Toxicity

Oral

The reported acute oral LD₅₀ values in rats for Benzethonium Chloride include 368, 420 ± 25, 450, and 665 mg/kg.^(60,135-138) These values place Benzethonium Chloride in the moderately and slightly toxic categories according to Hodge and Sterner.⁽¹³⁹⁾ In the tests conducted by Finnegan and Dienna,⁽⁶⁰⁾ a few deaths occurred within 24 hours, but approximately half of the animals died after 1 week; the longest survival period was 21 days. All deaths were preceded by severe depression.

Rosen et al.⁽¹³⁸⁾ conducted a study to determine the influence of dimethyl-

sulfoxide (DMSO) on the permeability and absorption of Benzethonium Chloride and other drugs. The acute oral LD_{50} s for Benzethonium Chloride in both distilled water and in 50 percent DMSO were determined in rats and mice. Male albino rats of the Charles River CD strain and Charles River CD-1 albino male and female mice were used. All animals were fasted 18 hours prior to oral dosing; a constant volume of 16 ml/kg, previously determined to be well tolerated, was used for both mice and rats. A total of 220 mice and rats were used: approximately 10 animals per dose group with 5 or 6 dose groups in each experiment. The acute oral LD_{50} s for Benzethonium Chloride administered in water and DMSO, respectively, were 665 and 368 mg/kg in rats and 485 and 338 mg/kg in mice. The lower values (more toxic) obtained with use of DMSO were statistically significant ($p < 0.05$). The investigators concluded that DMSO used as a solvent increased the oral toxicity of Benzethonium Chloride in both rats and mice.

Subcutaneous

Mason et al.⁽¹⁴⁰⁾ subcutaneously injected 5 groups of 4 Fischer 344 rats with varying doses of Benzethonium Chloride; the acute LD_{50} was 119.0 mg/kg.

Gall⁽¹²⁶⁾ tested Benzethonium Chloride for adjuvant activity with a purified diphtheria toxoid. A 0.2 ml dose of 1 Lf in borate-succinate buffer was mixed with 0.1 mg Benzethonium Chloride, gently agitated for 1 hour on a turntable, and then subcutaneously injected into the abdominal wall of 5 albino guinea pigs. Twenty-eight days later, the animals received a second injection of the diphtheria toxoid alone. Diphtheria antitoxin titers were measured by the guinea pig intracutaneous method. Benzethonium Chloride was a moderately active adjuvant. No mention was made of any specific toxic effects due to the administration of Benzethonium Chloride alone.

Intraperitoneal

Finnegan and Dienna⁽⁶⁰⁾ reported an intraperitoneal LD_{50} of 33.1 ± 2.5 mg/kg in male albino rats. Death usually occurred in 24 hours and was preceded by severe depression.

The lowest intraperitoneal lethal doses in mice were 7.813⁽¹⁴¹⁾ and 8 mg/kg.⁽¹³⁵⁾

Intravenous

The acute intravenous toxicity of Benzethonium Chloride in mice was studied by Arro and Salenstedt.⁽¹²²⁾ Twofold dilutions of a 10 percent Benzethonium Chloride solution were prepared, and 0.5 ml of each was injected into 5 mice. Five control animals were administered the diluent, Parker's medium 199. Observation continued for 14 days. The mean intravenous LD_{50} in 2 experiments was 35 mg/kg.

The intravenous LD_{50} of Benzethonium Chloride in male albino rats was 19.1 ± 0.8 mg/kg. Most of the animals died within 10 minutes, although a few deaths occurred after several hours. All deaths were preceded by severe depression. Hematuria was noted in these animals immediately after dosing, but erythrocyte numbers determined at 48 hours were only slightly lower than normal.⁽⁶⁰⁾

Intranasal

Benzethonium Chloride was tested in vitro and in vivo for activity against influenza A virus.⁽¹⁴²⁾ Four mice under light ether anesthesia were inoculated intranasally with 0.05 ml of a mixture of equal volumes of Benzethonium Chloride and virus in saline. Deaths and the degree of lobar consolidation of the lungs were recorded for the next 10 days. Benzethonium Chloride at a concentration of 0.0125 percent completely inhibited influenza A virus (no deaths or lobar consolidation), and 0.00625 percent gave partial inhibition (no deaths but presence of some lobar consolidation). These concentrations of Benzethonium Chloride were above the toxic range for mice. Concentrations of Benzethonium Chloride ranging from 0.25 to 4 percent were initially tested also but were toxic, causing death with lobar consolidation similar to an influenzal pneumonia.

In the in vivo methods, lightly etherized mice were exposed to Benzethonium Chloride preceding and following the test virus dose of 10 MLD. Preliminary doses were administered into the intranasal passage by means of a pasteur pipet; however, subsequent doses were administered via a spray chamber. Five mice were exposed to 2 percent Benzethonium Chloride for 9 minutes before and after the 10 minute virus dose. Five additional mice were exposed only to the test virus dose (in the same chamber) as controls. This was repeated with 5 mice exposed to 0.4 percent Benzethonium Chloride for 60 minutes before and after the virus dose. In both cases, Benzethonium Chloride had no protective activity: 2 and 3 mice of the 2 percent Benzethonium Chloride group died on Days 6 and 7, respectively, whereas 2 and 3 of the 0.4 percent group died on Days 5 and 7, respectively. Based on these results and those of a similar study on Sephiran and pneumococcus, Benzethonium Chloride appears to be rapidly inactivated upon contact with the lung.⁽¹⁴²⁾

Irritation

Topical

A percutaneous toxicity study was conducted by Finnegan and Dienna⁽¹³⁷⁾ to determine the local and systemic effects of Benzethonium Chloride and Methylbenzethonium Chloride. The hair of 12 healthy albino rabbits was clipped from the back and sides over an area extending from the neck to the hind legs. Two milliliters of a 0.1 percent solution of Benzethonium Chloride were applied to the clipped area of the skin of 6 rabbits once daily, 5 days per week for 4 weeks. Similarly, a 0.1 percent solution of Methylbenzethonium Chloride was applied to the other 6 rabbits. The animals were closely watched for signs of irritation, but none were observed during this period.

Groups of 3 male C57BL/6 mice (black mice) were given single doses of 8.75, 17.5, 35, 70, 140, and 280 mg/kg Benzethonium Chloride (in tricaprylin) by application with a camel's hair brush. A control group was painted with tricaprylin alone. Severe local blistering occurred at the 2 highest doses, more moderate local reactions occurred at the 70 and 35 mg/kg doses, and no visible reactions occurred at the 2 lowest doses. The mice had no immediate bleaching. Ten days later, spotty depigmentation occurred at the site of painting in 2 of the mice of the 140 mg/kg group. Hair exposed in vitro for 48 hours was not bleached by the same concentrations of Benzethonium Chloride as used in this experiment.⁽¹³⁰⁾

Ocular

Benzethonium Chloride and Methylbenzethonium Chloride were evaluated for ocular irritancy by an "irritant threshold" test.⁽¹³⁷⁾ The test solutions were introduced into the conjunctival sac of the rabbit eye, and observations of edema, erythema, and increased secretions were recorded for 1 hour. Five rabbits were used at each concentration (not specified) within a selected significant range. The threshold concentration, defined as the highest concentration not producing irritation in 3 or more of the 5 test rabbits was determined for each compound. Benzethonium Chloride and Methylbenzethonium Chloride were quite irritating, with a threshold concentration of 0.03 percent.

A Draize eye irritation test was conducted on Benzethonium Chloride. Three groups of 3 albino rabbits each received a 0.1 ml instillation of Benzethonium Chloride in distilled water into the conjunctival sac of 1 eye; the other eye served as the control. In the first group of 3 rabbits, the treated eye was not rinsed. The treated eyes of the second and third groups were rinsed with 20 ml of lukewarm water 2 and 4 seconds after instillation, respectively. Reactions were recorded at 24, 48, and 72 hours and 4 and 7 days following treatment or until such time as all signs of irritation had disappeared. Benzethonium Chloride had a maximum tolerated concentration, defined as that concentration at which no corneal or iridic lesions are present at the seventh day reading, of 0.5 percent.⁽¹⁴³⁾

In a subchronic eye irritation study, a 0.1 percent solution of Benzethonium Chloride was instilled into the conjunctival sac of rabbit eyes 2 to 3 times per day for 1 to 3 months. Only the superficial layers of the cornea and conjunctiva were affected: the corneal epithelium was thick and rough, with slight vascularization of the corneal stroma. The deep layers of the cornea and the intraocular tissues were not damaged, as determined by slit lamp or microscopic examination.⁽¹⁴⁴⁾

A cologne stick containing 0.5 percent Benzethonium Chloride was evaluated for ocular irritation in 3 rabbits. A 0.1 g sample of the cologne was instilled into 1 eye of each rabbit; the other eye served as the control. Eyes were scored according to Draize at 1 hour and daily thereafter for up to 7 days. The highest daily scores for each rabbit were 4, 4, and 6 (max, 110); all eyes were normal by Days 4, 4, and 5, respectively. The cologne stick was minimally irritating according to the Draize standard of classification.⁽¹⁴⁵⁾

Intracutaneous

An intracutaneous irritation study was conducted on four quaternaries, including Benzethonium Chloride.⁽¹³²⁾ Intracutaneous injections of 0.1 ml of serial 3-fold dilutions of each compound were administered to rabbits. Corresponding dilutions of each compound, as well as the diluent (Parker's medium 199) alone, were injected on the same side in the same rabbit, and 4 rabbits were used for each of the 6 dilutions. Rabbits were observed daily for erythema and infiltration, and observations were recorded on Days 1, 2, 4, 6, and 13. Numerical scores of 0, 1, 2, and 3 were given for no visible reaction, slight, moderate, and severe reactions, respectively. All inoculations with the diluent alone produced no skin reactions. A concentration of 1.0 percent Benzethonium Chloride produced a reaction of grade 1 on Days 1 and 2, 3 on Days 6 and 13; 0.33 percent Benzethonium Chloride produced a reaction of grade 0.5 on Day 1, 1 on Day 2, and 3 on Days 6 and 13; 0.11 percent Benzethonium Chloride produced a reaction of grade 0 on Day 1, 0.1 on Days 2, 6, and 13; 0.037 percent Benzethonium Chlo-

ride produced a reaction of grade 0 on Days 1 and 6 and 0.5 on Days 2 and 13.

The investigators ranked the four compounds tested for relative toxicity. Benzethonium Chloride received the score of 3 on a relative scale of 3:3:1:2. They also commented that this test was not very sensitive, as similar scores were obtained with all the 4 compounds tested, and they suggested that the rabbits could be interfering with the results by scratching of the injection sites.⁽¹³²⁾

Vaginal

A contraceptive foam containing 0.2 percent Benzethonium Chloride produced no signs of vaginal mucosal irritation after 10 and 15 injections in rabbits and dogs, respectively, over a 3-week period.⁽¹²⁶⁾

In a test for the British Family Planning Association, a contraceptive foam containing 0.2 percent Benzethonium Chloride was applied to the vaginas of 2 monkeys 5 times per week for 4 to 6 months. The monkeys had no signs of vaginal irritation.⁽¹²⁸⁾

Subchronic Toxicity

Oral

Oral doses of Benzethonium Chloride ranging from 1.13 to 35.58 mg/kg were administered to both rats and rabbits. Little or no toxic effects were noted at any dose except for the highest. Average body weight was reduced in rats receiving 35.58 mg/kg Benzethonium Chloride over a subchronic exposure period (experimental period unspecified).⁽¹³⁶⁾

Percutaneous

A percutaneous toxicity study was conducted on 0.1 percent solutions of Benzethonium Chloride and Methylbenzethonium Chloride. The hair of 12 albino rabbits was clipped from the back and sides over an area extending from the neck to the hind legs. Two milliliters of the Benzethonium Chloride solution were applied to the clipped area of the skin of 6 rabbits once daily 5 days per week for 4 weeks. Similarly, 2 ml of the Methylbenzethonium Chloride solution were applied to the other 6 rabbits. The animals were observed for signs of systemic toxicity, including weight loss. At termination, all the animals were killed, and representative tissues were examined microscopically. No systemic effects were noted for either compound.⁽¹³⁷⁾

Subcutaneous

Sixty Fischer 344 rats were separated into 5 groups of 6, 12, 24, 12, and 6 with equal numbers of males and females, and administered twice-weekly subcutaneous injections of Benzethonium Chloride in saline for 4 weeks.⁽¹⁴⁰⁾ The doses of Benzethonium Chloride were separated by quarter- or half-log intervals. The maximum tolerated dose (by repeated injections) was 3.0 mg/kg.

Groups of 8 C57BL/6 mice (black mice) were given subcutaneous injections of Benzethonium Chloride (in tricapylin) ranging from 17.5 to 180 mg/kg. The maximum tolerated dose for repeated injection was 35 mg/kg. At injection sites some large ulcers were found, and these healed within approximately 4 weeks. In all of the mice receiving 70 mg/kg Benzethonium Chloride, depigmentation of the fur near the site of injection was noted after 34 days. Five of the eight mice receiving 35 mg/kg had a similar spotty depigmentation. When the depigmented fur was plucked, the new hair growth was also depigmented.⁽¹³⁰⁾

Chronic Toxicity

Oral

A 1-year chronic oral feeding study was conducted using 9 adult mongrel dogs.⁽⁶⁰⁾ Benzethonium Chloride was mixed with Purina dog chow meal to give concentrations of 5, 100, and 500 ppm. Each diet was fed to 3 dogs for 1 year. All animals appeared well and gained weight during the test period. Prior to the start of the experiment and during the sixth and twelfth months, hemoglobin and complete blood counts were determined; all values were within normal limits. At termination, the dogs were necropsied, and the following organs were preserved for histopathological examination: heart, liver, lungs, thyroid, stomach, small intestine, cecum, large intestine, spleen, pancreas, kidneys, adrenals, and gonads. No gross or microscopic abnormalities were noted (Table 5).

A 2-year chronic oral feeding study was conducted using albino rats.⁽⁶⁰⁾ Benzethonium Chloride was mixed with finely ground Purina dog chow meal to give concentrations of 50, 200, 1000, 2500, and 5000 ppm. Sixty male and sixty female rats were divided into 12 groups of 10 (separated as to sex) and were individually housed. One group of each sex was fed one of the diets, and one served as the control group. Mortality was increased only at the 5000 ppm concentration between Weeks 10 and 30 in both males and females. Similarly, body weights were not significantly reduced ($p < 0.05$) except at the 5000 ppm concentration during the first week. The erythrocyte and differential white blood cell counts and the hemoglobin values taken during the 11th and 23rd months were within normal limits. All rats dying on test (not autolyzed) and all survivors at termination were necropsied, and the following organs were preserved for histopathological examination: heart, liver, lungs, thyroid, stomach, small intestine, cecum, large intestine, spleen, pancreas, kidneys, adrenals, and gonads. Microscopic examination was made on tissues from the survivors and those that died just prior to termination. One male (of 6 examined) at the 2500 ppm level, and two (of 3 examined) at the 500 ppm level had testicular atrophy. At the first necropsies, greatly distended ceca were observed in the higher dietary groups (1000, 2500, and 5000 ppm). This condition became progressively worse with increasing Benzethonium Chloride concentration and apparently occurred less than a week after treatment initiation. Thinning of the cecal wall, but no other abnormalities, were found at microscopic examination (Table 5).

Chronic (experimental period unspecified) oral doses of Benzethonium Chloride ranging from 1.13 to 35.58 mg/kg were administered to rats and rabbits. Few, if any, toxic effects were observed at doses other than the highest. A reduction in average body weight was noted in rats receiving a dose of 35.58 mg/kg Benzethonium Chloride⁽¹³⁶⁾ (Table 5).

Subcutaneous

A 1-year subcutaneous toxicity and carcinogenicity study was conducted on Benzethonium Chloride.⁽¹⁴⁰⁾ Groups of 20, 40, 60, and 80 Fischer 344 rats received approximately 0.25 ml doses of 0.1, 0.3, 1.0, and 3.0 mg/kg Benzethonium Chloride in saline, respectively, twice weekly for 52 weeks. Animals were held for observation another 6 months after treatment. Three controls were used: a vehicle control of twice weekly 0.25 ml saline injections (60 males and 60 fe-

TABLE 5. Chronic Toxicity

<i>Ingredient</i>	<i>Concentration and/or Dose</i>	<i>Length of Study</i>	<i>Species</i>	<i>Number of Animals</i>	<i>Results</i>	<i>References</i>
<i>Oral</i>						
Benzethonium Chloride	5, 100, and 500 ppm in the diet	1 year	Dog	3 in each conc. group	No gross or microscopic abnormalities	60
Benzethonium Chloride	50, 200, 1000, 2,500, and 5000 ppm in the diet	2 years	Rat	20 in each conc. group	Increased mortality and decreased body weight at 5000 ppm; thinning of cecal wall at 1000, 2500, and 5000 ppm; no other abnormalities	60
Benzethonium Chloride	1,125-35,576 mg/kg	Chronic, unspecified	Rat and rabbit	Unspecified	Few toxic effects at doses other than the highest; decreased average body weight in rats at highest dose	136
<i>Subcutaneous</i>						
Benzethonium Chloride in saline	0.1, 0.3, 1.0, and 3.0 mg/kg	1 year—twice weekly injections; observed for 18 months	Rat	80, 60, 40, and 20 in the high to low dose groups, respectively	14% reduction in weight gain at highest dose at 12 months, decreasing to 12% at 18 months; no other adverse effects	140
Benzethonium Chloride in saline	0.0034 and 0.034 mg	Single injection observed for 15 months	Mice	100 in each dose group	Slight decrease in highest dose female body weights, slight increase in mortality of high dose group; no significant compound-related effects	146

males), a negative control (60 males and 60 females), and a positive control using predetermined fixed doses of nickel sulfide (80 males and 80 females). Toxicity was determined by survival time, weight changes, and drug-related lesions. The animals were necropsied either at 12 or 18 months, as planned, and tissues from all spontaneous deaths, moribund rats, those rats with gross lesions or abnormal organ weights, and randomly selected rats were examined microscopically. Mortality for the first 12 months was only 1.5 percent, compared to 75 percent for the positive control. Mortality of Benzethonium Chloride-treated rats at 18 months was 7.5 percent, compared to 5.8 to 8.3 percent for the negative and vehicle controls and 90 percent for the positive controls. Benzethonium Chloride, at the highest dose, causes a 14 percent reduction in weight gain over 12 months compared to untreated and vehicle controls. The rats recovered body weight slightly by 18 months, with a reduction of 12 percent. Lower doses resulted in less reductions in body weight gains (Table 5).

Newborn Swiss mice were injected subcutaneously with 2 doses of Benzethonium Chloride, 1 set at 10 percent of the LD_{50} (0.0034 mg) and 1 set at the approximate LD_{50} (0.034 mg). A single injection was administered to each mouse in groups of 50 males and 50 females per dose. A saline vehicle control group and positive control groups of dibenz(a,h)anthracene (DBA) in both corn oil and saline were used. Injections were made at the base of the tail, and the needle was inserted along the hypodermal layer to the area of the neck and shoulders. The entire mouse population was afflicted with a respiratory condition at 15 weeks, causing some mortality. The animals were also treated with a DDT dust preparation at 15 and 19 weeks for sarcoptic mange mites. Animals were observed for 15 months and then sacrificed. Body weights were in the normal range. Females receiving the higher dose of Benzethonium Chloride had slightly decreased body weights. No mortality trends were noted. However, the higher dose Benzethonium Chloride group had more deaths than did the high dose DBA group. Survival did not appear to be correlated with compound administration. No compound-related nonneoplastic lesions were noted at termination. An insignificant number of tumors were found (except for injection site tumors in positive controls) distributed among test and control groups. These were typical spontaneous tumors in mice, and no correlation could be made between Benzethonium Chloride treatment and neoplasm formation⁽¹⁴⁶⁾ (Table 5).

Teratogenesis

A series of studies designed to determine the teratogenic effect of Benzethonium Chloride in rats were conducted by Gilman and DeSalva.⁽¹⁴⁷⁾ Pregnant rats were administered doses of Benzethonium Chloride up to 35.58 mg/kg per day by gastric intubation on Days 6 through 15 of gestation. Animals were killed on Day 20, and the fetuses were examined. The high dose (35.58 mg/kg per day) of Benzethonium Chloride produced lower mean body weights and delayed ossification. This finding was confirmed in a second study. No clinical manifestations of skeletal deformity were observed in rat fertility, perinatal, and postnatal studies. The investigators concluded that delayed ossification was not an expression of skeletal teratogenic changes but was most likely related to maternal toxicity and secondarily to reduced fetal maturation.

The Chemical Evaluation Committee (CEC) of the National Toxicology Program (NTP) has recommended testing Benzethonium Chloride for reproductive effects.⁽¹⁴⁸⁾

Mutagenesis

The Ames test with *Salmonella typhimurium* strains TA1535, TA1537, TA1538, TA98, and TA100 was used to study the mutagenic potential of Benzethonium Chloride; assays were conducted both with and without rat liver S-9 fraction. Benzethonium Chloride, at a maximum dose of 100 nmol/plate (due to bacterial toxicity), was nonmutagenic both with and without metabolic activation.⁽¹⁴⁹⁾

Benzethonium Chloride was nonmutagenic in the NTP mutagenicity testing program using *S. typhimurium*.⁽¹⁵⁰⁾

Carcinogenesis

One hundred C57BL/6 male mice were injected subcutaneously in the groin with 0.7 mg Benzethonium Chloride (in tricapylin). Five weeks later, the injection sites were excised, minced in 6 ml of Ringer's solution, and pooled. The resulting mix was injected subcutaneously into 25 C57BL/6 mice of the same age. All animals were killed after 18 weeks and examined grossly and microscopically for tumors. Positive dibenzo(a,i)pyrene and negative (tricapylin) controls were also subjected to the same treatment. No significant mortality was observed in the Benzethonium Chloride-treated group. The injection sites had granulation tissue and multiple granulomas with numerous giant cells. Scar tissue and numerous cysts, lined by single layers of endotheliumlike cells or granulation tissue with numerous giant cells, frequently contained cholesterol clefts. Some cysts were filled with granular or reticular amorphous material. None of the fibroblasts in this granulation and scar tissue had features suggestive of malignant transformation. Similar foreign body reactions were observed in the negative controls. All of the mice receiving Benzethonium Chloride had local depigmentation of the fur. However, depigmentation was not seen in any of the secondary hosts. Benzethonium Chloride was not found to be carcinogenic under these test conditions.⁽¹³⁰⁾

In another study, reportedly "the most sensitive system" for carcinogen detection, 0.35 mg Benzethonium Chloride were injected as a single dose into the tail vein of each of 50 CF-1 and 50 A/Jax female mice. Twenty additional CF-1 female mice were given 7 injections at monthly intervals. Positive dibenzo(a,i)pyrene and negative (Ringer's solution) controls were used for the single dose groups. All mice were killed at 7 months. The lungs were inflated with formaldehyde and examined under a dissecting microscope for tumors visible on the lung surface. Benzethonium Chloride did not induce a significant number of pulmonary tumors when compared to controls.⁽¹³⁰⁾

In a cocarcinogen study, 50 C57BL/6 male mice were each injected subcutaneously with 12.5 µg dibenzo(a,i)pyrene (DBP) in tricapylin. Twenty-four hours and again 8 days later, 0.70 mg of Benzethonium Chloride was injected into the same site. An additional series of Benzethonium Chloride at one-half the maxi-

imum tolerated dose, 0.35 mg/mouse, was started approximately 6 weeks later. This group received 0.35 mg Benzethonium Chloride 1, 8, and 15 days after the injection of DBP. Positive (croton oil) and negative controls were used. All animals in the 0.70 mg group were killed at 29 weeks; those in the 0.35 mg group at 23 weeks. Tissues from all were examined microscopically for tumors; tumors found were fibrosarcomas produced by DBP. Benzethonium Chloride was not cocarcinogenic. However, the study was considered inconclusive due to the fact that croton oil, the positive control, failed to have any cocarcinogenic action. The investigator commented on the "significant" inhibiting effect of Benzethonium Chloride upon tumor formation following the injection of DBP. Benzethonium Chloride groups had 34.1 and 0 percent cumulative tumor incidence for the high and low dose groups, respectively, compared to 48.8 and 52.0 percent for the positive control (croton oil and DBP) and the DBP control. All mice receiving 0.7 mg Benzethonium Chloride and 97.4 percent of the 0.35 mg Benzethonium Chloride group had depigmented fur.⁽¹³⁰⁾

Newborn Swiss mice were injected subcutaneously with 2 doses of Benzethonium Chloride, a low dose (0.0034 mg) set at 0.1 of the LD₅₀ and a high dose set at the approximate LD₅₀ (0.034 mg). A single injection was administered to each mouse of groups of 50 males and 50 females per test dose. Positive (DBA) and vehicle (saline) control groups were used. Animals were observed for 15 months, killed, and tissues were examined microscopically. No compound-related nonneoplastic lesions were noted at termination. An insignificant number of tumors was found distributed among the test and control groups (with the exception of injection site tumors in positive controls). These were typical spontaneous tumors for mice, and no correlation could be made between Benzethonium Chloride treatment and neoplasm formation. Under these study conditions, Benzethonium Chloride was not a chemical carcinogen.⁽¹⁴⁶⁾

Weanling Swiss and Balb/c male and female mice were to be given multiple injections of Benzethonium Chloride (concentration not given) either subcutaneously or intraperitoneally every 2 weeks for 20 injections. Difficulties ensued so that only 15 to 16 injections were given. Animals were observed for 18 months. Similar groups of mice were given a single injection of Benzethonium Chloride as controls. A significant number of tumors did not occur in the treated mice as compared to controls. Benzethonium Chloride was not carcinogenic in these mice.⁽¹⁵¹⁾

In a carcinogenicity study, groups of 20, 40, 60, and 80 (equal males and females) Fischer weanling rats were injected subcutaneously with 0.1, 0.3, 1.0, and 3.0 mg/kg Benzethonium Chloride in saline, respectively, twice weekly for 52 weeks.⁽¹⁴⁰⁾ Animals were held for observation another 6 months after treatment. Positive (nickel sulfide), negative, and vehicle (saline) controls were used. All rats were necropsied at the time of death or at the 18-month sacrifice and tissues were examined microscopically for tumor formation. The treated rats had 26 sarcomas at injection sites (13 percent) compared to 0 and 1 tumors in the vehicle and negative controls (0 to 2 percent) and 90 percent incidence in the positive controls. A high incidence of granulomatous reactions occurred at the sites of the subcutaneous injection of Benzethonium Chloride, and these were dose related. The tumors were principally fibrosarcomas; none metastasized, but some did grow to a large size. This type of induced neoplasm has been described as arising from mesenchymal cells in the area of repeated irritation. Based on the incidence

of dose-related tumors at the injection sites, Benzethonium Chloride should be classified as a weak carcinogen under the classification system of Grasso and Goldberg.⁽¹⁵²⁾ Clayson,⁽¹⁵³⁾ however, regards the induction of localized sarcomas in mice upon repeated subcutaneous injection of test solutions as "notoriously unreliable as an indicator of carcinogenicity." Furthermore, he considers "the results of individual experiments as extremely variable."

A 2-year chronic oral feeding study was conducted using albino rats.⁽⁶⁰⁾ Benzethonium Chloride was mixed in the diet to give concentrations of 0, 50, 200, 1000, 2500, and 5000 ppm. One group of 10 males and one of 10 females were fed each dietary concentration. Rats that died and those killed at termination were necropsied and tissues were examined microscopically. Three mammary gland fibroadenomas were found; none occurred in the 2 highest dose groups. The investigators commented that this tumor occurrence of 6.5 percent was low for rats of that age. One subcutaneous reticulum cell sarcoma was found in a male of the 200 ppm group after 53 weeks, but the occurrence of this tumor was unrelated to treatment because no such tumors were found in higher dose groups over a longer period of time.

The Chemical Evaluation Committee of the NTP has recommended Benzethonium Chloride for carcinogenicity testing.⁽¹⁵⁰⁾

CLINICAL ASSESSMENT OF SAFETY

Irritation and Sensitization

An occlusive patch containing 0.1 ml of a 5 percent aqueous Benzethonium Chloride solution was applied to the upper back of each of 100 volunteer white males. Patches were removed after 48 hours, and the sites were evaluated 0, 1, 24, 48, and 72 hours later. Fifty-one of the 100 subjects had irritant reactions, defined as "redness without vesiculation or infiltration, declining in intensity 24 hours after removal, nonitching, and not spreading beyond the patch." In doubtful cases, the patches were repeated on the forearms. The investigators noted that Benzethonium Chloride is a known irritant, not a sensitizer⁽¹⁵⁴⁾ (Table 6).

In clinical studies designed to determine the antiseptic properties of Benzethonium Chloride, observations were made on skin sensitivity or other toxic reactions. The conditions of the test conformed to hospital preoperative skin preparation procedures. The abdominal skin of each patient was first scrubbed with a tincture of green soap and water, cleansed with alcohol, and then painted with either an aqueous solution of Benzethonium Chloride (0.2 percent) or a tincture of Benzethonium Chloride (0.2 percent) on one side only. Three hundred obstetric deliveries and 100 surgical cases were evaluated. The skin of each patient was carefully observed for several days after treatment; no evidence of irritation, desquamation, or other reactions was noted. A tincture of Benzethonium Chloride was further studied to determine the effect of heat and sweating on its antiseptic properties. Both thighs of 10 patients were painted with the Benzethonium Chloride tincture and a heat cradle was placed over 1 thigh of each for 2½ hours. No irritation or rise in bacterial count was noted⁽¹⁵⁵⁾ (Table 6).

An aerosol antiperspirant and a deodorant, each containing 0.12 percent Benzethonium Chloride, were evaluated in a repeated insult patch test for irrita-

TABLE 6. Clinical Irritation and Sensitization

<i>Ingredient</i>	<i>Type of Test</i>	<i>Number of Humans</i>	<i>Results/Comments</i>	<i>References</i>
Benzethonium Chloride in 5% aqueous solution	48-hour occlusive patch	100	51 exhibited irritant reactions, "redness without vesiculation or infiltration, declining in intensity 24 hours after removal, nonitching, and not spreading beyond the patch"; irritant	154
Benzethonium Chloride in 0.2% aqueous solution or tincture of green soap and water	Single topical application on scrubbed abdominal skin (hospital preoperative skin preparation procedures)	400	No irritation, desquamation, or other reactions noted	155
Benzethonium Chloride in 0.2% tincture of green soap and water	Single topical application on thighs with heat administered for 2½ hours	10	No irritation	155
Benzethonium Chloride, 0.12% in an aerosol antiperspirant	RIPT*	50	No reactions; no effects indicative of a primary irritant, fatiguing agent, or sensitizer	156
Benzethonium Chloride, 0.12% in a deodorant	RIPT	50	No reactions; no effects indicative of a primary irritant, fatiguing agent, or sensitizer	157
Benzethonium Chloride, 0.1% in an aerosol foam	Clinical test for symptomatic control in patients with dermatological disorders; applications every 2 hours for 6 days (average)	98	One case of slight stinging with first application in a patient with pruritis; no other side effects; nonirritant	158
Methylbenzethonium Chloride, 0.5% in a skin cleanser	Irritation—single patch	18	No reactions; no difference in irritancy between product and control; nonirritant	159
Methylbenzethonium Chloride, 0.5% in a skin cleanser	RIPT with rechallenge test	100	A total of 10 barely perceptible or mild (1+ on scale of 0–4) reactions observed in 7 subjects during induction and in 6 subjects at challenge; 3 subjects were rechallenged, resulting in 1 mild reaction considered due to the abrasive nature of the cleanser; investigators concluded the cleanser did not induce sensitization; mild irritant; nonsensitizer	160

*RIPT, repeated insult patch test.

tion and sensitization.^(156,157) Each product (full strength) was applied using a 24-hour occlusive patch on 50 human subjects. Each site was examined at the time of patch removal and graded on a scale of 0 to 4, representing no response to a severe response. Patches were applied Monday through Thursday for 2 weeks, followed by a 2-week rest period and a challenge patch application to the same area in the fifth week of the study. Challenge patches were removed after 24 hours, and the sites treated with the aerosol antiperspirant were examined at removal and after 24, 48, and 96 hours. Sites treated with the deodorant were examined 10 minutes, 24, 48, and 72 hours after removal of the patches. All 50 subjects in each test had no reactions during the induction or challenge phases. The investigators concluded that, under these test conditions, neither product was a primary irritant, fatiguing agent, or sensitizer in any of the subjects. They also predicted with 95 percent certainty that 92.89 percent of a general population would not be sensitized by these products (Table 6).

A hydrocortisone-containing aerosol foam with 0.1 percent Benzethonium Chloride was tested clinically for symptomatic control in 98 patients with dermatological disorders. These consisted of contact dermatitis (70), pruritis ani (14), neurodermatitis (6), and miscellaneous disorders (8). In most cases, the aerosol was applied every 2 hours for an average time of 6 days. With the exception of 1 case of slight stinging with the first application in a patient with pruritis, no other side effects were reported⁽¹⁵⁸⁾ (Table 6).

A skin cleanser containing 0.5 percent Methylbenzethonium Chloride was tested for skin irritation in 18 human subjects.⁽¹⁵⁹⁾ A patch containing a 0.1 ml dose of the cleanser was applied to the volar surface of the forearm or inner aspect of the arm. A comparable product was used as a control. Reactions were graded 2 and 24 hours after patch removal on a scale of 0 to 4, representing no response to a severe response. All 18 panelists had no reactions. Type of patch, application, and duration of application were unspecified. The control product was also nonirritating (Table 6).

A skin irritation and allergic contact sensitization test was conducted to evaluate a cleanser containing 0.5 percent Methylbenzethonium Chloride.⁽¹⁶⁰⁾ Twenty-four hour occlusive patches were applied to the backs of 100 panelists (96 females, 4 males). Each patch contained a 0.1 ml dose of the full-strength cleanser, and applications were made every Monday, Wednesday, and Friday at the same site for 3 consecutive weeks. Responses were scored on a scale of 0 to 4 just prior to applications 2 through 9 and the next test date after application 9. Following a 2-week nontreatment period, a 24-hour challenge patch was applied to a previously unpatched site, and reactions were scored 24 and 48 hours after removal. Any subjects with reactions at challenge indicative of possible induced sensitization were retested 1 week later. This follow-up testing consisted of occlusive patches with the cleanser full-strength and in a 1:3 dilution, and an example of exaggerated use in which the cleanser was applied full-strength to the flex area of the arm 3 times daily for 5 days. A total of 10 reactions were observed in 7 subjects during the induction phase: 5 barely perceptible and 5 mild. Six subjects had a total of 10 reactions at challenge, 4 of whom had no reactions during induction. The reactions observed were mild (1+) or barely perceptible, and 3 of these subjects were selected for rechallenge. All 3 subjects gave no response 24, 48, and 72 hours after the full strength and diluted patch applications. One subject had a mild reaction on Day 3 of the 5-day exaggerated use test. However,

this subject had been vigorously rubbing the "abrasive cleanser" onto her arm, causing noticeable abrasions considered irritant in nature. The majority of reactions during induction and challenge were apparently due to this same grain pressure effect. This reactivity was not confirmed in the 3 rechallenged subjects. Under these test conditions, the cleanser had no potential for inducing sensitization (Table 6).

Fisher⁽¹⁶¹⁾ reported that Benzethonium Chloride is rarely a sensitizer to either skin or oral membranes. Topical oral application may produce allergic sensitization.

Case Reports

Three cases of Benzethonium Chloride sensitization have been reported. A laborer using Benzethonium Chloride and benzalkonium chloride applied topically as disinfectants for many years developed a contact dermatitis. On patch testing, he was sensitive to both compounds in dilutions of 1:100,000.⁽¹⁶²⁾ A 23-year-old woman acquired a severe edematous, pruritic, vulvar and perivulvar eruption after using a hygiene spray containing Benzethonium Chloride for 1 month. This was superimposed upon a seborrheic dermatitis in the inguinal area. A 1:1000 aqueous solution of both Benzethonium Chloride and benzalkonium chloride produced a strongly positive reaction in this patient. A 29-year-old man developed penile and scrotal dermatitis after each act of sexual intercourse with a woman who used a spray containing Benzethonium Chloride. This man also gave strongly positive reactions to patch tests with 1:1000 aqueous solutions of Benzethonium Chloride and benzalkonium chloride.⁽¹⁶¹⁾ Fisher suggests that individuals may have cross sensitization reactions to Benzethonium Chloride and benzalkonium chloride.

Irritation—Ocular, Vaginal, Oral, and Otic

Benzethonium Chloride is used as a preservative in ophthalmic preparations. Over a period of 5 years, various wetting agents, including Benzethonium Chloride, were administered to "hundreds" of patients at an eye clinic. Instillations varied from a single administration to 2 to 4 times daily for periods up to 3 years. The cornea were examined by slitlamp, and patients were questioned regarding irritation. Solutions of 0.1 percent administered as a single 0.50 ml drop into the conjunctival sac of volunteers produced conjunctival reactions consisting of hyperemia, edema, and thickening and reduced transparency of the superficial layers associated with dilation of capillaries. Other effects noted were profuse lacrimation, enlargement of deeper-lying vessels in some cases, and frequently, desquamation of the conjunctival epithelium. Corneal lesions developed 10 minutes after instillation. Solutions of approximately 0.03 to 0.04 percent instilled 3 to 4 times daily for 2 to 8 weeks produced similar but much less severe corneal reactions. In most cases, recovery time was 12 hours or less. The investigator reported that individuals had varied responses to these solutions but that the minimal concentrations producing irritation were greater than those found therapeutically effective. Surface activity, pH variation, and osmotic pressure were cited as possible causes of irritation. Frequent or prolonged administration greatly increased the severity of the irritation.⁽¹⁴⁴⁾

Benzethonium Chloride has been added to several ophthalmic solutions used to reduce intraocular pressure. A carbostyryl derivative containing 0.1 mg

Benzethonium Chloride was placed in the eye of a person with secondary glaucoma, and no irritation was reported.⁽¹⁶¹⁾ Bupranolol solutions (35 μ l of 1 percent or 0.5 percent) containing 0.01 g Benzethonium Chloride were instilled in 1 eye of each of 12 normal humans and 30 patients with either ocular hypertension or primary open-angle glaucoma. The other eye of the 12 served as controls, whereas the other eye of the 30 received a placebo instillation. No side effects were reported. Similarly, 10 patients received 4 times daily instillations of the 1 percent Bupranolol solution for 6 months. Two of the patients also received a 1 percent pilocarpine solution. No side effects were reported, with the exception of a stinging sensation at the time of instillation and lasting for several seconds. At the end of 6 months, no change in isopters or abnormalities in tear secretions were found.⁽¹⁶⁴⁾

In a review of Benzethonium Chloride as a preservative in ophthalmic preparations, the Ophthalmic Panel of the FDA OTC Drug Review Program found Benzethonium Chloride satisfactory as a preservative at maximum concentrations of 0.01 percent for preparations used directly in the eye and 0.02 percent for preparations not for direct use in the eye.⁽¹⁷¹⁾

A number of studies have been conducted on a vaginal aerosol foam contraceptive containing 0.2 percent Benzethonium Chloride and 8.0 percent nonyl phenoxypolyethoxyethanol in an oil in water dispersion. One hundred thirty women of childbearing age used the foam over an average period of 20.4 months (1 to 57 months), and no clinical evidence of irritation, sensitization, or other abnormalities resulted.⁽¹⁶⁵⁾ Sobrero⁽¹⁶⁶⁾ encountered no reports of burning, itching, irritation, or unfavorable reactions by husbands to the foam in postcoital tests with 22 women. He also found that daily vaginal injections in 12 volunteer women over approximately 3 weeks produced no evidence of irritation by inspection or papanicolaou smear. In another study, 142 couples used the foam for a month or more; 1 couple discontinued the test because of urethral irritation in the wife.⁽¹²⁸⁾ Six percent (15 of 247) of the volunteers in a trial of the foam complained of postcoital irritation.⁽¹⁶⁷⁾ A study was conducted involving 2932 women who used the foam over an average period of 270 days (1076 used the foam for 1 year or more). Of the 1994 women who discontinued the study, 107 did so because of vaginal irritation and 17 discontinued because of irritation of the genitalia of the male partner. There were no reports of severe local reaction.⁽¹⁶⁸⁾ (Table 7).

A 3-year trial of a spermicidal cap containing 4.5 mg Benzethonium Chloride and 39.0 mg nonoxynol 9 was conducted with 326 couples, of which 230 couples completed the test. Average use was 14 months. Mild redness, burning, and local irritation occurred in 18 couples (5.5 percent) and caused one couple to discontinue the test. Variation in vaginal pH and inflammatory changes were not of clinical significance. No evidence of neoplastic changes was found in cells of the portio or of the vagina in cytologic preparations obtained from these areas.⁽¹⁶⁹⁾

In a study of odor changes following the intravaginal administration of a suppository containing 0.4 mg Methylbenzethonium Chloride to 10 women, no vaginal irritation was reported.⁽¹⁷⁰⁾

Numerous clinical studies have been conducted to determine the effect of antimicrobial formulations containing Benzethonium Chloride on the onset of caries, calculus, and gingival inflammation. Benzethonium Chloride generally re-

TABLE 7. Clinical Vaginal Irritation

<i>Ingredient</i>	<i>Type of Test</i>	<i>Number of Humans</i>	<i>Results/Comments</i>	<i>References</i>
Benzethonium Chloride, 0.2% in an aerosol foam contraceptive	Normal use for an average period of 20.4 months	130 women	No evidence of irritation, sensitization, or other abnormalities	163
Benzethonium Chloride, 0.2% in an aerosol foam contraceptive	Postcoital test	22 couples	No reports of burning, itching, irritation, or unfavorable reactions by husbands	165
Benzethonium Chloride, 0.2% in an aerosol foam contraceptive	Daily vaginal injection for 3 weeks	12 women	No evidence of irritation	128
Benzethonium Chloride, 0.2% in an aerosol foam contraceptive	Normal use for 1 or more months	142 couples	1 couple discontinued test due to urethral irritation in wife	166
Benzethonium Chloride, 0.2% in an aerosol foam contraceptive	Postcoital trial	247 women	15 complaints of irritation	167
Benzethonium Chloride, 0.2% in an aerosol foam contraceptive	Normal use for an average period of 270 days	2932 women	107 cases of irritation; 7 cases of irritation to male genitalia; no instances of severe local reactions	168
Benzethonium Chloride, 4.5 mg in a spermicidal cap	Normal use for an average period of 14 months	230 couples	Mild redness, burning, and local irritation in 18 couples, causing 1 to discontinue the test; no evidence of neoplastic changes in cells of the portio or vagina	169
Methylbenzethonium Chloride, 0.4 mg in a vaginal suppository	Single application	10 women	No vaginal irritation	170

duced plaque accumulation but had no significant effect on gingivitis. No signs of oral mucosal irritation were reported.^(98-99, 101, 103)

In several studies otic solutions containing approximately 0.02 percent Benzethonium Chloride were used both prophylactically and in the treatment of otitis externa. Drops were instilled in the ears of a total of 1282 humans as frequently as 4 times daily and for periods up to 16 days. No irritation or other toxic effects were reported.⁽¹⁷¹⁻¹⁷⁵⁾

SUMMARY

Benzethonium Chloride and Methylbenzethonium Chloride are synthetic quaternary ammonium salts occurring as colorless to white, slightly odorous, and bitter tasting monohydrate crystals. Both compounds are soluble in water, the lower alcohols, glycols, ether, and benzene. The exact methods of manufacture of these compounds are proprietary. Commercial preparations contain a large number of functional groups and impurities that contribute to the physicochemical properties of the product. Analytical procedures for the detection and quantification of Benzethonium Chloride and Methylbenzethonium Chloride are numerous, and most depend on the formation of a relatively stable ion-pair complex.

Benzethonium Chloride and Methylbenzethonium Chloride are relatively stable compounds. Their reactivity is determined for the most part by their cationic properties. They are inactivated by and adsorbed onto a variety of substances.

Benzethonium Chloride and Methylbenzethonium Chloride are used in cosmetics primarily as preservatives and secondarily as cationic surfactants, usually at concentrations below 1 percent. In 1981, Benzethonium Chloride and Methylbenzethonium Chloride were used in 93 and 33 formulations, respectively. Cosmetic products containing these compounds may contact all external body surfaces and hair, as well as ocular and vaginal mucosae. Frequency and length of application could result in continuous exposure. Benzethonium Chloride and Methylbenzethonium Chloride also are used widely in industry and in pharmaceuticals and are indirect food additives (limited to use as preservatives in adhesives).

Benzethonium Chloride and Methylbenzethonium Chloride have been extensively used and studied as antimicrobials. Controversy surrounds the scope of their microbial spectrum and their inactivation by a large number of materials. They are considered more bactericidal than fungicidal, and their antimicrobial activity has been credited generally to their ability to disrupt permeability of cell membranes. Benzethonium Chloride and Methylbenzethonium Chloride cause additional cytolytic injury by protein denaturation, by oxidation and enzyme inhibition, by effects on activating ions, and by interference with growth and reproduction. Tissue effects of Benzethonium Chloride include weakened activity of cilia of mucosal cells of the isolated trachea of the mouse and inhibition of smooth muscle contraction in rabbit ileum.

Fur depigmentation was noted in black mice either receiving Benzethonium Chloride injections or painted with the compound.

In acute toxicity studies, Benzethonium Chloride was moderately to slightly toxic when administered orally or intraperitoneally and mildly toxic to mice treated intranasally with concentrations of 0.25 to 4 percent. The subcutaneous LD₅₀ for Benzethonium Chloride was 119.0 mg/kg in rats and the intravenous LD₅₀ was 35 and 19.1 mg/kg in mice and rats, respectively.

Benzethonium Chloride and Methylbenzethonium Chloride were both irritating to the rabbit eye at concentrations greater than 0.03 percent. Additionally, Benzethonium Chloride produced ocular irritation in rabbits at a concentration of 0.1 percent and gave a maximum tolerated concentration (no corneal or iridic lesions at 7 days) of 0.5 percent. A cologne stick containing 0.5 percent Benzethonium Chloride was minimally irritating to rabbit eyes.

Both Benzethonium Chloride and Methylbenzethonium Chloride were non-irritating when applied topically (0.1 percent) to the skin of rabbits, although mice had local reactions to the moderate and high doses (35 to 280 mg/kg) of Benzethonium Chloride in a skin-painting study. Benzethonium Chloride was slightly irritating when administered intracutaneously to rabbits and nonirritating in tests of vaginal contraceptives with rabbits, dogs, and monkeys.

In subchronic studies, little or no toxic effects were found when Benzethonium Chloride was administered orally or percutaneously. Methylbenzethonium Chloride was also nontoxic when administered percutaneously. The maximum tolerated dose of Benzethonium Chloride administered subcutaneously was 3.0 and 35 mg/kg in rats and mice, respectively.

Chronic oral studies were also generally negative for toxic effects due to Benzethonium Chloride except for a slight increase in mortality and reduced body weight in rats at the high dietary concentration (5000 ppm) in one study. Greatly enlarged ceca were also noted in this latter study. In 1 of 2 chronic subcutaneous studies on Benzethonium Chloride, reduced weight gain was found at the high dose only, and a 13 percent incidence of sarcomas at injection sites was dose related.

Placental transport of Benzethonium Chloride was variable and inconsistent. Some delayed ossification was noted in fetuses. However, this change was attributed to maternal toxicity and only secondarily to reduced fetal maturation.

Benzethonium Chloride was nonmutagenic in a microbial sensor system and in the Ames test both with and without metabolic activation. No evidence of carcinogenicity of Benzethonium Chloride was found in 6 studies with mice and rats. However, in a seventh study, Benzethonium Chloride was classified as a weak carcinogen in rats due to a high incidence (13 percent) of compound-related tumors at injection sites. Benzethonium Chloride was not cocarcinogenic in combination with 3,4,9,10-dibenzpyrene (DBP) in mice, and in fact had a significant inhibiting effect upon tumor formation following injection of DBP.

In clinical studies, Benzethonium Chloride produced mild skin irritation at 5 percent and at lower concentrations produced no irritation. An aerosol antiperspirant and a deodorant, each containing 0.12 percent Benzethonium Chloride, produced no irritation or sensitization reactions when tested in human volunteers. Two skin cleansers, each containing 0.5 percent Methylbenzethonium Chloride, were tested for irritation, and irritation and sensitization, respectively. No irritation resulted from use of the first cleanser; irritant reactions caused by the second were apparently due to the "abrasive" nature of the cleanser, and no

sensitization was noted with either product. Three cases of Benzethonium Chloride sensitization have been documented.

Benzethonium Chloride is used as a preservative in ophthalmic preparations with maximum concentrations of 0.1 percent for use directly in the eye and 0.02 percent for use not directly in the eye. In various clinical uses of ophthalmic wetting agents containing Benzethonium Chloride, individuals varied in their irritant response to these solutions, although a general dose-response was seen. Increasing irritation was observed at concentrations greater than those found antibacterially effective.

In numerous studies of a vaginal aerosol foam contraceptive containing 0.2 percent Benzethonium Chloride, no irritation was found in 3 and no sensitization in 1. In others 0.7, 4, and 6 percent irritation were reported. Methylbenzethonium Chloride produced no vaginal irritation when used in a suppository. Benzethonium Chloride (4.5 mg) in a spermicidal cap produced mild irritation in 5.5 percent of the test population after an average use period of 14 months. No neoplastic changes were found in cells of the portio or vagina of these women.

No signs of oral mucosal irritation were noted in studies with antimicrobial formulations containing Benzethonium Chloride. Also, no irritation or toxic effects were produced by otic solutions containing Benzethonium Chloride.

DISCUSSION

Benzethonium Chloride and Methylbenzethonium Chloride are weak antimicrobial agents. They can be irritating to the skin at concentrations of 5 percent or greater. However, at the concentrations used in cosmetics, neither compound produces significant irritation. These compounds have been reported to be ocular and vaginal irritants. In one study, fur depigmentation was noted in black mice upon subcutaneous injection or topical application of Benzethonium Chloride.

CONCLUSION

On the basis of the available animal and clinical data, the Expert Panel concludes that Benzethonium Chloride and Methylbenzethonium Chloride are safe at concentrations of 0.5 percent in cosmetics applied to the skin. A maximum concentration of 0.02 is safe for cosmetics used in the eye area.

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REFERENCES

1. JOSLYN, D.A., YAW, K., and RAWLINS, A.L. (1943). Germicidal efficacy of phemerol. *J. Am. Pharm. Assoc. Sci. Ed.* 32, 49-51.
2. ESTRIN, N.F., CROSLEY, P.A., and HAYNES, C.R. (eds.) (1982). *CTFA Cosmetic Ingredient Dictionary*, 3rd ed. Washington, DC: Cosmetic, Toiletry and Fragrance Association.
3. UNITED STATES PHARMACOPEIA (USP). (1979). 20th ed. Rockville, MD: United States Pharmacopeial Convention, Inc.
4. HAWLEY, G.G. (ed.). (1971). *Condensed Chemical Dictionary*, 8th ed. New York: Van Nostrand Reinhold.
5. HAZLETON, L.W. (May 1952). Relation of surface active properties to irritation of the rabbit eye. *Proc. Sci. Sec., Toilet Goods Assoc.*, No. 17.
6. WINDHOLZ, M. (ed.). (1976). *The Merck Index*, 9th ed. Rahway, NJ: Merck and Co.
7. COSMETIC, TOILETRY and FRAGRANCE ASSN. (CTFA). (Oct. 8, 1981). Submission of unpublished data by CTFA. Cosmetic ingredient chemical description, Benzethonium Chloride (2-15-12).*
8. CTFA. (Oct. 8, 1981). Submission of unpublished data by CTFA. Cosmetic ingredient chemical description, Methylbenzethonium Chloride (2-15-13).*
9. CTFA. (March 23, 1983). Submission of unpublished data by CTFA. UV absorbance data on Benzethonium Chloride and Methylbenzethonium Chloride.*
10. CTFA. (March 28, 1983). Submission of unpublished data by CTFA. UV Absorbance data on Benzethonium Chloride.*
11. GRANT, J. (ed.). (1972). *Hack's Chemical Dictionary*, 4th ed. New York: McGraw-Hill.
12. JAPAN COSMETIC INDUSTRY ASSOCIATION (JCIA). (1979). *Japanese Standards of Cosmetic Ingredients*, 1st ed. Tokyo, Japan: Yakuji Nippo, Ltd.
13. AMERICAN PHARMACEUTICAL ASSOCIATION (APA). (1974). National Formulary Board. *National Formulary XIV*. Washington, DC: American Pharmaceutical Association.
14. JUNGEMANN, E. (1979). Fat-based surface-active agents, in: D. Swern (ed.) *Bailey's Industrial Oil and Fat Products*, 4th ed. New York: Wiley, Vol. 1, pp. 642-3.
15. GERSTEIN, T. (Nov. 1979). An introduction to quaternary ammonium compounds. *Cosmet. Toilet.* 94, 33-41.
16. GOSSELIN, R.E., HODGE, H.C., SMITH, R.P., and CLEASON, M.N. (1976). *Clinical Toxicology of Commercial Products*, 4th ed. Baltimore, MD: Williams & Wilkins.
17. TANAKA, K., HIOKI, M., and SHINDO, H. (1974). Determination of pancuronium bromide and its metabolites in human urine by dye-extraction method - relation between the extractability and structure of quaternary ammonium ions. *Chem. Pharm. Bull. (Jpn.)* 22(11), 2599-606.
18. ROSEN, M.J. (1978). *Surfactants and Interfacial Phenomena*. New York: Wiley.
19. CAMERON, J. (1974). Preservative systems compatible with DPT-Polio (Salk) and TABTD-Polio (Salle) vaccines. *Dev. Biol. Stand.* 24, 155-65.
20. ROSOFF, I.S. (1974). *Handbook of Veterinary Drugs*. New York: Springer.
21. FOOD AND DRUG ADMINISTRATION. (FDA). (Dec. 12, 1980). Establishment of a monograph and proposed rulemaking on vaginal contraceptive drug products for over-the-counter human use. *Fed. Reg.* 45(241), 82017, 82042.
22. SCHIMMEL, J., and SLOTSKY, M.N. (1974). Preservation of Cosmetics, in: M.S. Balsam and E. Sagarin (eds.), *Cosmetics Science and Technology* 3, 391-470. New York: Wiley.
23. AMERICAN MEDICAL ASSOCIATION (AMA). (1977). *AMA Drug Evaluations*, 3rd ed. Chicago, IL, p. 890.
24. MOORE, K.E., and STRETTON, R.J. (1978). Detection of preservatives in pharmaceutical and cosmetic products using agar gel electrophoresis. *J. Chromatogr.* 156(1), 211-7.
25. SENZEL, A.J. (ed.). (1977). *Newburger's Manual of Cosmetic Analysis*, 2nd ed. Washington, DC: Assoc. of Official Analytical Chemists.
26. BALEUX, B., and CAUMETTE, P. (1977). Biodegradation of some cationic surfactants. *Water Res.* 11(9), 833-42.
27. OLSON, B.H., ELDERING, G., and GRAHAM, B. (1964). Stabilization of pertussis vaccine in the presence of Benzethonium Chloride. *J. Bacteriol.* 87, 543-6.

*Available upon request: Administrator, Cosmetic Ingredient Review, Suite 810, 1110 Vermont Avenue, N.W., Washington, DC 20005

28. BERSET, C., JASKIEWICZ, H., TRAN MANH SUNG, G., and SANDRET, F. (1979). Electrolytic purification methods for waste water. Elimination of synthetic surfactants. *Trib. Cebedean (French)* 32(424), 79.
29. TSURUBOU, S., OHNO, N., and SAKAI, T. (1980). Extraction-spectrophotometric determination of benzethonium in the presence of quaternary ammonium salts with 2,6-dibromophenolindophenol. *Nippon Kagaku Kaishi (Jpn.)* 6, 828-32.
30. SAKAI, T., TSUBOUCHI, M., and ZAKI, Y. (1976). Spectrophotometric determination of cationic surfactants with 2,6-di-chloroindophenol by solvent extraction. *Bunseki Kagaku (Jpn.)* 25(10), 675-9.
31. BIANCHI, W., BONIFORTI, L., and DI DOMENICO, A. (1974). Analysis of some quaternary ammonium compounds by gas chromatography-mass spectrometry. *Mass Spectrom. Biochem. Med. Symp.*, pp. 183-96.
32. ALARY, J., ROCHAT, J., VILLET, A., and COEUR, A. (1976). Microdetermination of organic bases by atomic absorption. I. Assay of quaternary ammonium [compounds]. *Ann. Pharm. Fr. (French)* 34(9-10), 345-53.
33. HIROSE, S., YOSHIDA, S., and ATOMORI, M. (1977). Determination of a disinfectant by complementary tristimulus colorimetry and diphasic titration. *Yakuzaigaku (Jpn.)* 37(1), 38-45.
34. PINZAUTI, S., LA PORTA, E., PAPERESCHI, G., and BIFFOLI, R. (1980). A mercury-coated platinum electrode in the direct potentiometric determination of quaternary ammonium salts. *J. Pharm. Belg.* 35(4), 281-4.
35. PINZAUTI, S., and LA PORTA, E. (1977). Use of the silver electrode in the potentiometric determination of quaternary ammonium compounds. *Analyst (London)* 102(1221), 938-42.
36. PINZAUTI, S., and LA PORTA, E. (1979). Potentiometric analysis with a silver electrode in stability control of quaternary ammonium salt disinfectant solutions during storage in plastic and glass containers. *J. Pharm. Pharmacol.* 31(2), 123-4.
37. PINZAUTI, S., and LA PORTA, E. (1979). The potassium ion selective electrode as a tetraphenylborate sensor for quaternary ammonium salts analysis. *J. Pharm. Pharmacol.* 31(8), 573-4.
38. JOHNSON, E. (1978). Reverse phase ion pair chromatography of high molecular weight quaternary amines. *Ind. Res. Dev.* 20(6), C₂, C₄.
39. LOWRY, J.B. (1979). Direct spectrophotometric assay of quaternary ammonium compounds using Bromthymol Blue. *J. Pharm. Sci.* 68(1), 110-1.
40. AHREND, K.F., and TIESS, D. (1973). Thin-layer chromatographic parameters of 180 toxicologically significant compounds with low volatility in four simple systems with instructions for practical application. *Wiss. Z. Univ. Rostock, Math.-Naturwiss. Reihe (German)* 22(9), 951-63.
41. TACHIZAWA, M. (1976). Use of mixed fluorescent materials for thin-layer chromatographic drug analysis. *Iyakuhi Kenkyu (Jpn.)* 7(4), 570-7.
42. HOSOBUCHI, K., and SATO, K. (1978). Effect of gamma-irradiation on disinfectants. 4. Benzalkonium Chloride, Benzethonium Chloride, and Cetylpyridium Chloride cationic surfactants. *Bokin Bobai* 6(4), 154-60.
43. KAO SOAP CO. (Jan. 1980). Stable Quaternary Ammonium Chloride Compositions. *Jpn. Kokai Tokkyo Koho Patent No.* 80-07219.
44. HART, J.R., and LEVY, E.F. (1977). Sarcosinate-cationic creme rinse shampoo. *Soap Cosmet. Chem. Spec.* 53(8), 31-4, 60B-60C.
45. BOUCHAL, A.W. (Jan. 19, 1960). US Patent 2,921,855. Colgate-Palmolive Co.
46. REYBROUCK, G. (1979). Efficacy of inactivators against 14 disinfectant substances. *Zentralbl. Bakteriell. Parasitenkd. Infektionskr. Hyg. Abt. 1: Orig. Reihe B.* 168(5-6), 480-92.
47. MYBURGH, J.A., and MCCARTHY, T.J. (1980). Inactivation of preservatives in the presence of particulate solids. *Pharm. Weekbl. Sci. Ed.* 2(5), 137-42.
48. FDA. (Dec. 1981). Product formulation data. Computer printout.
49. CODE OF FEDERAL REGULATIONS (CFR). (1979). Title 21, Part 720.4. Washington, DC.
50. EUROPEAN ECONOMIC COMMUNITY (EEC). (June 15, 1982). List of preservatives which cosmetic products may contain. Annex 4, Annex VI, of a Council Directive of May 17, 1982, amending Directive 76/768/EEC. *Off. J. Eur. Communities* No. L167/1-31.
51. DREWE, N.W., PARKER, R.J., and TADROS, T.F. (Aug. 2, 1973). Herbicidal quaternary bipyridylum salts. *Ger. Offen. Patent No.* 2304204 (Imperial Chemical Industries).
52. FORD, R.R., and TADROS, T.F. (Aug. 21, 1975). Herbicidal compositions containing bipyridylum salts. *Ger. Offen. Patent No.* 2506834 (Imperial Chemical Industries).
53. YAMASHITA, S., TSUYUKI, H., FURUKAWA, T., KIWATA, T., and HISAYAMA, H. (Jan. 12, 1979). Germicide composition. *Jpn. Kokai Tokkyo Koho Patent No.* 79 04287 (Tokyo Organic Chemical Industries).

54. UENO, S., and UENO, Y. (Feb. 3, 1978). Luminous deodorant, disinfectant, and insecticidal compositions for toilets. Jpn. Kokai Patent No. 7812432.
55. KOSE, Y., KAWAMURA, H., OZAWA, M., and OHTSUKA, K. (Jan. 24, 1973). Slow-release pesticide. Jpn. Patent No. 73 02331 (Sankyo Co.).
56. KOSE, Y., KAWAMURA, H., OZAWA, M., and OHTSUKA, K. (Jan. 26, 1973). Sustained release type pesticide. Jpn. Patent No. 73 02775 (Sankyo Co.).
57. GOLDHAFT, T.M., KAIRZ, C., and MAIER, G.D. (May 10, 1977). Disinfectant composition comprising a quaternary ammonium compound, a phenol, and formaldehyde. U.S. Patent No. 4022911 (Damon Corp.).
58. EGUCHI, Y., and IJIMA, E. (Jan. 30, 1980). Benzethonium disinfectants. Jpn. Kokai Tokkyo Koho Patent No. 80 13228 (Kao Soap Co.).
59. SHIRAHATA, A., NOZAKI, Y., HORIUCHI, T., ISHIKAWA, K., MUTO, M., MIZUTANI, K., OIKAWA, T., MIZUHARA, H., and MAEDA, N. (1975). Investigations of bacterial infection rate and bacterial flora of premature infants during no gowning period. *Acta Paediat. Jpn.* 17(1), 31.
60. FINNEGAN, J.K., and DIENNA, J.B. (1954). Toxicity of quaternaries. *Soap Sanit. Chem.* 30, 147, 149, 152-3, 157, 173, 175.
61. PIVNICK, H., TRACY, J.M., and GLASS, D.G. (1963). Studies of preservatives of poliomyelitis (Salk) vaccine. I. Benzethonium Chloride. *J. Pharm. Sci.* 52, 883-8.
62. TSUKADA, K., and KATO, W. (Aug. 18, 1979). Stable injections of high concentrations of DOPA and related compounds. Jpn. Kokai Tokkyo Koho Patent No. 79105221 (Sankyo Co.).
63. TOMIBE, K., OHTSU, A., KONDO, N., and SUENAGA, E. (Dec. 2, 1976). Production of heparin derivatives. Jpn. Kokai Patent No. 76139884 (Teijin).
64. HASTINGS, S.G., POSCHEL, B.P.H., and BUTLER, D.E. (Dec. 6, 1977). Treating cardiovascular disorders. US Patent No. 4061756 (Parke, Davis and Co.).
65. CHRISTIE, G.J., and BUCKWALTER, F.H. (July 22, 1975). Ketamine hydrochloride-containing anesthetic. US Patent No. 3896221 (Bristol-Myers Co.).
66. BURNAP, R.W. (April 12, 1977). Anesthetic and sedative composition. US Patent No. 4017619.
67. BACHMAYER, H., and REEVE, P. (Feb. 1, 1979). Subunit Vaccines. Ger. Offen. Patent No. 2829089 (Sandoz-Patent-G.m.b.H.).
68. SHEWMAKE, S.W., and ANDERSON, B.C. (1979). Hydrofluoric acid burns: A report of a case and review of the literature. *Arch. Dermatol.* 115(5), 593-6.
69. REINHARDT, C.F., HUME, W.G., LINCH, A.L., and WEATHERHOLD, J.M. (1966). Hydrofluoric acid burn treatment. *Am. Ind. Hyg. Assoc. J.* 27, 166-71.
70. CHECCHI, A.A. (1982). *OTC Drug Ingredient Index and Manual*. Washington, DC. pp. 92.0-7; 92A-1.0; 2.0, 404.0-3; 570.0-3.
71. FDA. (May 6, 1980). Establishment of a monograph and proposed rulemaking on ophthalmic drug products for over-the-counter human use. Fed. Reg. 45(89), 30005-6, 30017.
72. FDA. (Sept. 13, 1974). Establishment of monograph and use of certain halogenated salicylanilides as active or inactive ingredients. OTC Topical Antimicrobial Products and Drug and Cosmetic Products. Fed. Reg. 39(179), 33116, 33131-2.
73. FDA. (Jan. 6, 1978). Tentative final monograph on over-the-counter drugs generally recognized as safe, effective, and not misbranded. OTC Topical Antimicrobial Products. Fed. Reg. 43(14), 1218, 1227, 1229-30, 1236-37.
74. FDA. (March 28, 1980). Establishment of a monograph on antacids drug products for over-the-counter human use: proposed rulemaking. Fed. Reg. 45(62), 20670.
75. FDA. (Nov. 7, 1980). Proposed rulemaking on hair grower and hair loss prevention. Drug products for over-the-counter human use. Fed. Reg. 45(218), 73957.
76. CFR. (1979). Title 21, Part 175.105(G)(5). Washington, DC.
77. KUMAR, A., and NARASIMHAN, R. (1977). Ripening in sugar cane with Polaris, Cetrinide, and Hyamine 1622. *Indian Sugar* 26(12), 817-20.
78. KUMAR, A., and NARASIMHAN, R. (1977). Ripening in sugar cane with Polaris, Cetrinide, and Hyamine 1622. *Indian Sugar* 27(8), 437-41.
79. FDA. (Nov. 13, 1980). Status of ingredients in the OTC drug review. Division of OTC Drug Evaluation (HFD-510), Bureau of Drugs.
80. FDA. (Dec. 4, 1979). Establishment of a monograph and notice of proposed rulemaking on external analgesic drug products for over-the-counter human use. Fed. Reg. 44(234), 69772.
81. CFR. (1979) Title 21, Part 210.3(b)(7). Washington, DC.
82. CFR. (1979) Title 21, Part 210.3(b)(8). Washington, DC.

83. FELDKAMP, C.S., EPSTEIN, E., THIBERT, R.J. and ZAK, B. (1975) Spectrophotometric study of drug interferences in an aqueous 17 ketosteroid reaction. *Microchem. J.* 20(4), 523-33.
84. IWATA, J., and NISHIKAZE, O. (July 1979) New microturbidimetric method for determination of protein in cerebrospinal fluid and urine. *Clin. Chem.* 25(7), 1317-9.
85. WHYMAN, A.E. (Feb. 1970) Measurement of tritium in neat plasma and urine with a Toluene/Triton X-100/Hyamine 10-X Scintillant. *Int. J. Appl. Radiat. Isot.* 21(2), 81-6.
86. STEDMAN, R.L., KRAVITZ, E., and KING, J.D. (1957) Studies on cell surface-germicide and enzyme-germicide reactions and their contribution to the lethal effect. *J. Bact.* 73, 655.
87. HARVEY, C., and STUCKEY, R.E. (1962) Spermicidal activity of surface active agents. *Reprod. Fert.* 3, 124-31.
88. LAWRENCE, A. (1950) *Surface-Active Quaternary Ammonium Germicides*. New York: Academic Press, pp. 113-26.
89. ROSENTHAL, M.W. (1972) Mouthwashes. in M.S. Balsam and E. Sagarin (eds.) *Cosmetics: Science and Technology*, 2nd ed. New York: Wiley, Vol. I, Chap. 15, pp. 533-63.
90. NADA, T., ITO, H., YAMAMOTO, H., IMAI, J., and TAMURA, T. (1980) Bacteriostatic and bactericidal effects of ordinary disinfectants against glucose-nonfermenting gram-negative rods. *Eisei Kensa* 29(7), 929-37.
91. GOTO, S., KANEKO, Y., KAWASAKI, K., ANJO, S., KAGAMI, M., SATO, T., OKURA, M., MIYOSHI, Y., and MIYASHITA, S. (1977) Bactericidal activity of conventional disinfectants against clinically isolated strains of gram-negative rods. *Rinsho Byon* 25(8), 684-90.
92. NAITO, R., ITOH, T., HASEGAWA, E., ARIMURA, H., FUJITA, Y., HASEGAWA, K., INABA, T., KAGI-TANI, Y., KOMEDA, S., MATSUMOTO, T., OKAMOTO, H., OKANO, K., AGUKO, Y., and OGUSHI, T. (1974) Bronopol as a substitute for thimerosal. *Dev. Biol. Stand.* 24, 39-48.
93. CHRISTENSEN, P.E. (1963) Evaluation of the antibacterial effect of preservatives, with special preference to phemerol and thimerosal. *Acta. Pathol. Microbiol. Scand.* 57, 104-10.
94. RAWLINS, A.L., SWEET, L.A., and JOSLYN, D.A. (1943) Relationship of chemical structure to germicidal activity of a series of quaternary ammonium salts. *J. Am. Pharm. Assoc. Sci. Ed.* 32, 11-6.
95. NIKKO CHEMICALS K.K. (April 1, 1981) Antimicrobial formulations with acylated peptides. *Jpn. Kokai Tokkyo Koho Patent No.* 81 32420.
96. KAWAMURA, M., TAKEDA, K., SHIMADA, T., and YAMAMOTO, Y. (1980) Synergistic effect of Hyamine-T solution and Hibitane Gluconate in controlling *Pseudomonas*, *E. coli* and *S. aureus*. *Byon Yaku-gaku* 6(2), 127-33.
97. ITO, K., KITAMURA, J., and NOMURA, T. (1975) The effect of polyene antibiotics on the permeability of the cell membrane of *Candida albicans* and its polyene-resistant mutant *euphoria cacophoria*. 2, 20-31.
98. VOLPE, A.R., KUPEZAK, L.J., BRANT, J.H., KING, W.J., KESTENBAUM, R.C., and SCHLISSEL, H.J. (1969) Antimicrobial control of bacterial plaque and calculus and the effects of these agents on oral flora. *J. Dent. Res.* 48, 832-41.
99. COMPTON, F.H., and BEAGRIE, G.S. (1975) Inhibitory effect of Benzethonium and Zinc Chloride mouthrinses on human dental plaque and gingivitis. *J. Clin. Periodontol.* 2, 33-43.
100. TANZER, J.M., SLEE, A.M., KAMAY, B., and SCHEER, E.R. (1979) In vitro evaluation of seven cationic detergents as antiplaque agents. *Antimicrob. Agents Chemother.* 15(3), 408-14.
101. GOMER, R.M., HOLROYD, S.V., FEDI, P.F., and FERRIGNO, P.D. (March-April 1972) The effect of oral rinses. *J. Am. Soc. Prevent. Dent.* 2, 7, 12, 55, 58.
102. GAFFAR, A., MARCUSSEN, H.W., SOLIS-GAFFAR, M.C., and RUSTOGI, K.N. (1980) Long term anti-plaque gingivitis and calculus effects of Benzethonium Chloride in beagle dogs. *J. Periodont. Res.* 15(1), 107-10.
103. CARTER, H.G., and BARNES, G.P. (May-June, 1975) Effects of three mouth-washes on existing dental plaque accumulations. *J. Prev. Dent.* 2(3), 6-8, 10-11.
104. BAKER, P.J., COBURN, R.A., GENCO, R.J., and EVANS, R.T. (1978) The in vitro inhibition of microbial growth and plaque formation by surfactant drugs. *J. Periodont. Res.* 13(5), 474-85.
105. COMPTON, F.H., BEAGRIE, G.S., CHERNECKY, R., and GLASSER, K. (1979) Effect of two antibacterial mouth sprays and dentifrices on dental plaque and gingivitis in beagle dogs. *J. Dent. Res.* 58(5), 1471-7.
106. ERIKSEN, H.M., JEMTLAND, B., FINCKENHAGEN, H.J., and GJERMØ, P. (1979) Evaluation of extrinsic tooth discoloration. *Acta Odontol. Scand.* 37, 371-5.
107. GAFFAR, A., and GRECEK, J.J. (Sept. 23, 1980) Antibacterial oral composition. U.S. Patent No. 4224309 (Colgate-Palmolive Co.)
108. GAFFAR, A., and VOLPE, A.R. (Nov. 23, 1978) Antibacterial composition for mouth care. Ger. Offen Patent No. 2722187 (Colgate-Palmolive Co.)

109. GAFFAR, A., and VOLPE, A.R. (Jan. 30, 1979) Antibacterial oral composition. US Patent No. 4137303 (Colgate-Palmolive Co.).
110. MOTE, E.M., SCHOESSLER, O.D., and HILL, R.M. (March 1969) Lens incorporated germicides. *J. Am. Optom. Assoc.* 40, 291-3.
111. GARDNER, R.A., and PITTMAN, M. (1965) Relative stability of pertussis vaccine preserved with merthiolate, Benzethonium Chloride, or the parabens. *Appl. Microbiol.* 13(4), 564-9.
112. WETTERLOW, L.H., and EDSALL, G. (1970) The effect of Benzethonium Chloride on the antigens of pertussis vaccine. *Int. Arch. Allergy Appl. Immunol.* 37(2), 143-53.
113. RIEGER, F., TSUJI, S., and MASSONLIS, J. (1972) Formes Natives et Globulaires de l'acetylcholinesterase Dans la Moelle epiniere et le Cerveau de Gymnote. *Electrophorus electricus. Eur. J. Biochim.* 30, 73.
114. MARKS, N., D'MONTE, B., and LAJTHA, A. (1973) Inhibition of cerebral proteolytic enzymes. *Texas Rep. Biol. Med.* 31, 345-65.
115. CALANDRA, J.C., HARDT, L.L., and STANISH, E.S. (1951) The effect of various compounds on trypsin activity. *Gastroenterology* 19, 5645.
116. BOEHME, D.H., UMEZAWA, H.L., HASHIM, G., and MARKS, H. (1978). Treatment of experimental allergic encephalomyelitis with an inhibitor of cathepsin (peptatin). *Neurochem. Res.* 3(2), 185-94.
117. AKOPYAN, T.N., ARUTUNYAN, A.A., LAJTHA, A., and GALOYAN, A.A. (1978). Acid proteinase of hypothalamus purification. Some properties, and action on somatostatin and substance P. *Neurochem. Res.* 3(1), 89-99.
118. MAKINEN, K.K. (1968). Inhibition of arylaminopeptidases and proline aminopeptidases of human whole saliva by Benzethonium Chloride Benzyl dimethyl (2-(2-(P-1,1,3,3-Tetramethylbutyl) Phenoxy)Ethoxy) Ethyl)Ammonium Chloride. *Febs Lett.* 2(2), 101-4.
119. SUGIURA, M., and OGISO, T. (1969) Enzymes. XLVIII. Bile-sensitive lipase. Effect of surfactants on Mucor Lipase. *Yakugaku Zasshi* 89(9), 1289-96.
120. CHEN, Y.T., ROSENBERRY, T.L., and CHANG, H.W. (1974) Subunit heterogeneity of acetylcholinesterase. *Arch. Biochem.* 161(2), 479-87.
121. JACKSON, R.L., and APRISON, M.H. (1966) Mammalian brain acetylcholinesterase. Effects of surface-active agents. *J. Neurochem.* 13(12), 1367-71.
122. NARA, T., TSUJI, K., and KOMATSU, T. (1974) Flavine 9. Effect of preservatives and hydroxybenzoic acid derivatives on the photodecomposition of flavine adenine dinucleotide. *Yakuzaigaku* 34(2), 65-71.
123. SAKURAI, T., MATSUMARU, H., and TSUCHIYA, S. (1977) Microenvironmental changes of thio-barbiturate molecules in protein binding. *Yakuzaigaku* 37(4), 169-75.
124. HURWITZ, A.R., and CARNEY, C.F. (1978) Enhancement of ampicillin partition behavior. *J. Pharm. Sci.* 67(1), 138-40.
125. HOLT, W.V., and DOTT, H.M. (1980). Chemically induced fusion between ram spermatozoa and avian erythrocytes: An ultrastructural study. *J. Ultrastruct. Res.* 71(3), 311-20.
126. GALL, D. (1966). The adjuvant activity of aliphatic nitrogenous bases. *Immunology* 11(4), 369-86.
127. KUWAHARA, K., HITOSHI, T., HAYASHI, H., and SATAKE, K. (1976). Effects of detergents on cultured human cells. *Kyushu Yakugakkai Kaiho.* 30, 31-7.
128. PANIAQUA, M.E., RIO PIEDRAS, P.R., VALLIANT, A.B., and GAMBLE, C.J. (1961). Field trial of a contraceptive foam in Puerto Rico. *JAMA* 177, 125.
129. BROTHERTON, J. (1977). Assessment of spermicides by a stripping technique against human spermatozoa. *J. Reprod. Fert.* 51, 383-97.
130. HOMBURGER, F. (1968). Carcinogenicity of several compounds. National Technical Information Service, PB. No. 183 027, 26 pp. —
131. SCHLEPPY, C.A., and ETTER, J.C. (1976). Applications of the ciliary activity test of the mouse trachea to evaluate components used to formulate nasal preparations. *Pharm. Acta Helv.* 51(10), 297-303.
132. ARRO, L., and SALENSTEDT, C.R. (1973) Evaluation of toxicity of some quaternary ammonium compounds. *J. Biol. Stand.* 1(1), 87-99.
133. NADAI, T., KUME, M., TATEMATSU, A., and SEZAKI, H. (1975). Drug-induced histological changes and its consequences on the permeability of small intestinal mucosa. II. *Chem. Pharm. Bull.* 23(3), 543-51.
134. LA ROSA, R.T., FINE, N., and DE SALVA, S.S. (1978) Rat maternal and fetal absorption of ¹⁴C-Benzethonium Chloride (¹⁴C-BTC). *Pharmacologist* 20, 254.
135. SAX, N.I. (1979) *Dangerous Properties of Industrial Materials*, 5th ed. New York: Van Nostrand Reinhold.
136. FDA. (Nov. 1979) Information copy of OTC topical antifungal report. Division of OTC Drug Evaluation (HFD-510). Bureau of Drugs.
137. FINNEGAN, J.K., and DIENNA, J.B. (1951) Toxicological observations on certain surface active agents. *Toilet Goods Assoc. Proc. Sci. Ser.* 20, 16-9.

- 138 ROSEN, H., BLUMENTHAL, A., PANASEVICH, R., and McCALLUM, J. (1965). Dimethylsulfoxide (DMSO) as a solvent in acute toxicity determinations. *Proc. Soc. Exp. Biol. Med.* 120, 511-4.
- 139 HODGE, H.C., and STERNER, H. (1949). Tabulation of toxicity classes. *Am. Indus. Hyg. Assoc. (Quart.)* 10, 93-6.
- 140 MASON, M.M., CATE, C.C., and BAKER, J. (1971). Toxicology and carcinogenesis of various chemicals used in the preparation of vaccines. *Clin. Toxicol.* 4(2), 185-204.
- 141 CHEMICAL BIOLOGICAL COORDINATION CENTER. (Feb. 1950). Summary biological tests. Washington, DC: National Research Council.
- 142 KLEIN, M., and STEVENS, D.A. (1945). The in vitro and in vivo activity of synthetic detergents against influenza A virus. *J. Immunol.* 50, 265-73.
- 43 DRAIZE, J.H., and KELLEY, E.A. (May 1952). Toxicity to eye mucosa of certain cosmetic preparations containing surface-active agents. *Toilet Goods Assoc. Proc. Sci. Sect.* 17, 1-4.
- 44 SWAN, K.C. (1944). Reactivity of the ocular tissues to wetting agents. *Am. J. Ophthalmol.* 27, 1118-22.
- 145 CTFA. (Aug. 16, 1983). Submission of unpublished data by CTFA. Rabbit eye irritation test.*
- 146 DERSE, P.H. (1970). Injection of Newborn Mice with Seven Chemical Adjuvants to Help Determine their Safety in Use in Biologicals. U.S. Clearinghouse Fed. Sci. Tech. Inform., PB Rep 195153:135 p. 1969 From U.S. Govt. Res. Develop. Rep. 70, 56.
- 147 GILMAN, M.R., and DeSALVA, S.J. (1979). Teratology studies of Benzethonium Chloride, Cetyl Pyridinium Chloride and Chlorhexidine in rats. *Toxicol. Appl. Pharmacol.* 48, A35.
- 148 NATIONAL TOXICOLOGY PROGRAM (NTP). (Aug. 1981). Technical Bulletin. Dept. of Health and Human Services, Public Health Service, 5.
- 149 DeFLORA, S. (1981). Study of 106 organic and inorganic compounds in the *Salmonella*/microsome test. *Carcinogenesis (London)* 2(4), 283-98.
- 150 NTP. (April 1983). Technical Bulletin. Dept. of Health and Human Services, Public Health Service, 9.
- 151 KIRSCHSTEIN, R.L. (1974). Toxicology and carcinogenicity of preservatives used in the preparation of biological products. *Dev. Biol. Stand.* 24, 203-12.
- 152 GRASSO, P., and GOLBERG. (1966). Subcutaneous sarcoma as an index of carcinogenic potency. *Food Cosmet. Toxicol.* 4, 297-320.
- 153 CLAYSON, D.B. (1962). *Chemical Carcinogenesis*. Boston, MA: Little, Brown, p. 341.
- 154 KLIGMAN, A.M., and LEYDEN, J.J. (1979). Reactions to standard patch test materials. *Acta Derm. Venerol. (Sweden)* 59(Suppl. 85), 101-3.
- 155 BROWN, W.E., GUNDERSON, M.F., SCHWARTZ, P., and WILDER, V.M. (1944). A clinical and bacteriological study of phemerol as a skin antiseptic. *Surg. Gynecol. Obstet.* 78, 173-80.
- 156 FOOD AND DRUG RESEARCH LABORATORIES (FDRL). (Sept. 10, 1973). Evaluation of potential hazards by dermal contact with an aerosol antiperspirant, sample RG-385-J (2-15-11).*
- 157 FDRL. (April 19, 1974). Evaluation of potential hazards by dermal contact with a deodorant concentrate, sample HRC-385-1 (2-15-10).*
- 158 WISE, R. (May 1968). Clinical trial of a unique hydrocortisone-containing topical aerosol in a variety of dermatologic disorders. *Ill. Med. J.* 133, 611-3.
- 159 CTFA. (Dec. 5, 1980). Submission of unpublished data by CTFA. Clinical skin irritation test (2-15-4).*
- 160 CTFA. (April 3, 1981). Submission of unpublished data by CTFA. Allergic contact sensitization test (2-15-5).*
- 161 FISHER, A.A. (1973). Allergic reaction to feminine hygiene sprays. *Arch. Dermatol.* 108(6), 801-2.
- 162 SHMUNES, E., and LEVY, E.J. (Jan. 1972). Quaternary ammonium compound contact dermatitis from a deodorant. *Arch. Dermatol.* 105, 91-3.
- 163 TANAKA, N., NISHINAKAMURA, Y., and NAKAGAWA, K. (Aug. 28, 1980). Agent for treating glaucoma. *Ger. Offen. Patent No.* 3001011 (Otsuka Pharmaceutical Co.).
- 164 TAKASE, M., KOMURO, S., NANBA, H., and ARAIE, M. (1978). Effects of topical bupranolol hydrochloride on the intraocular pressure. *Jpn. J. Ophthalmol.* 22(1), 142-54.
- 165 BUSHNELL, L.F. (1965). A practical and effective method of contraception. *Pacif. Med. Surg.* 73, 353.
- 166 SOBRERO, A.J. (1960). Evaluation of a new contraceptive. *Fertil. Steril.* 11, 518-24.
- 167 MEARS, E. (1962). Chemical contraceptive trial. II. *J. Reprod. Fertil.* 4, 337.
- 168 BERNSTEIN, G.S. (Jan. 1971). Clinical effectiveness of an aerosol contraceptive foam. *Contraception* 3, 37-43.
- 169 GIAROLA, A., PERNIOLA, L., GAZZANI, G., and MAGNI, E. (Nov. 1979). Long-term multicentre trial with TA-RO CAP, a new spermicidal product. *Contraception* 20(5), 489-95.
- 170 DRAVNIKS, A., KEITH, L., KROTOSZYNSKI, B.K., and SHAH, J. (Jan. 1974). Vaginal odors. GLC assay method for evaluating odor changes. *J. Pharm. Sci.* 63, 36-40.

- 171 HEILIG, D., HEILIG, M.C., and GLASSMAN, J.M. (Dec. 1979). Prophylactic use of a topical nonaqueous acetic acid medication for the prevention of otitis externa (swimmer's ear). Two year study with follow-up. *Curr. Ther. Res. Clin. Exp.* 26, 862-73.
- 172 ORDONEZ, G.E., KIME, C.E., UPDEGRAFF, W.R., GLASSMAN, J.M., and SOUKA, J.P. (May 1978). Effective treatment of acute, diffuse otitis externa. 1. Controlled comparison of hydrocortisone-acetic acid, nonaqueous and hydrocortisone-neomycin-polymyxin B otic solutions. *Curr. Ther. Res. Clin. Exp.* 23(5) [Suppl.], 3-14.
- 173 KIME, C.E., ORDONEZ, G.E., UPDEGRAFF, W.R., GLASSMAN, J.M., and SOYKA, J.P. (May 1978). Effective treatment of acute, diffuse otitis externa. 2. Controlled comparison of hydrocortisone-acetic acid, nonaqueous and hydrocortisone-neomycin-colistin otic solutions. *Curr. Ther. Res. Clin. Exp.* 23(5) [Suppl.], 15-28.
- 174 GARRITY, J.D., HALLIDAY, T.C., and GLASSMAN, J.M. (1974). Prevention of swimmer's ear by simple prophylactic regimen. *Curr. Ther. Res.* 16(5), 437-41.
- 175 DADACIAN, A.J., HICKS, J.J., ORDONES, G.E., and GLASSMAN, J.M. (1974). Treatment of otitis externa: A controlled bacteriological clinical evaluation. *Curr. Ther. Res.* 16(5), 431-6.

15(MISSING)

Toxicity to Eye Mucosa of Certain Cosmetic Preparations Containing Surface-Active Agents

by J. H. DRAIZE and ELSIE A. KELLEY*

ALTHOUGH surface active agents represent a relatively new development in the field of synthetic chemistry, at present more than a thousand such preparations are known. Such an agent is a substance which when added to a system of one or more interfaces has the property of orienting itself in such a manner as to act as a coupling agent bringing the interfaces in more intimate contact. The majority of surface active agents are not pure chemical compounds but are derivatives or a condensation of several compounds and are designated in many cases by a general empirical formula. The slight variations in composition or lack of exact chemical definition may account for variations in toxicological behavior between lots or batches of a given agent. Surface active agents have been classified into three broad groups depending on their ionization characteristics and behavior, namely, cationic, anionic and nonionic. Although all these agents have properties in common, some behave predominantly as emulsifiers, others as penetrating and wetting agents, some as detergent and foaming agents, and some even as germicidal agents. Because of these properties these agents are finding ever wider use in cosmetic preparations. Their use in such cosmetic preparations as hair shampoos, bath preparations, wave lotions, etc., has led to a number of serious eye injuries.

Technics of Tests on Eye Mucosa

In general the technics of tests as published by Draize, et al¹ for testing the irritancy of substances on the eye mucosa are followed. According to these technics the lesions or reactions observed in the cornea, iris and the palpebral and bulbar conjunctivae are scored separately. In this method of assigning numerical scores to the lesions observed, approximately 80 per cent of the weight is given to the cornea and iris, those structures of the eye seriously concerned with vision.

In testing cosmetic preparations for potential eye irritation nine albino rabbits are used. One-tenth milliliter (cc) of the test substance is instilled in the conjunctival sac of one eye, with the other eye serving as the control. In the case of the first three subjects the treated eye remains unwashed. Since washing the eye may or may not alleviate symptoms of injury, the six remaining subjects are divided into two equal groups. In the first of these groups eyes instilled with the test substance are washed with 20 ml. of luke warm water (approximately at body temperature) 2 seconds after treatment and in the second group the treated eyes are washed 4 seconds after instillation. The washing procedures are regarded as significant since it is important to know the effect of such a procedure, that is, whether it is detrimental or beneficial, and if beneficial to ascertain the extent of the benefit.

Ocular reactions are read with a hand slit lamp with readings made at 24, 48, 72 hours, and at 4 and 7 days after treatment or as long as residual injury persists. A preparation which has elicited corneal and iris lesions which have not

cleared by the seventh day reading is considered a severe eye irritant. The cornea is scored on the basis of density of

TABLE I
Scale For Scoring Ocular Lesions¹

I. Cornea	
A. Opacity-Degree of Density (area which is most dense is taken for reading)	
Scattered or diffuse area-details of iris clearly visible....	1
Easily discernible translucent areas, details of iris slightly obscured	2
Opalescent areas, no details of iris visible, size of pupil barely discernible	3
Opaque, iris invisible	4
B. Area of Cornea Involved	
One quarter (or less) but not zero	1
Greater than one quarter-less than one half.....	2
Greater than one half-less than three quarters.....	3
Greater than three quarters up to whole area	4
A x B x 5	Total Maximum = 80
II. Iris	
A. Values	
Folds above normal, congestion, swelling, circumcorneal injection (any or all of these or combination of any thereof), iris still reacting to light (sluggish reaction is positive)	1
No reaction to light, hemorrhage, gross destruction (any of these or all of these)	2
A x 5	Total possible maximum = 10
III. Conjunctivae	
A. Redness (refers to palpebral conjunctivae only)	
Vessels definitely injected above normal	1
More diffuse, deeper crimson red, individual vessels not easily discernible	2
Diffuse beefy red	3
B. Chemosis	
Any swelling above normal (includes nictitating membrane)	1
Obvious swelling with partial eversion of lids	2
Swelling with lids about half closed	3
Swelling with lids about half closed to completely closed	4
C. Discharge	
Any amount different from normal (does not include small amounts observed in inner canthus of normal animals)	1
Discharge with moistening of the lids and hairs just adjacent to the lids	2
Discharge with moistening of the lids and considerable area around the eye	3
Score (A + B + C) x 2	Total Maximum = 20

The total score for the eye is the sum of all scores obtained for the cornea, iris, and conjunctivae.

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opacity and its total area involved. The iris is scored on the intensity or degree of inflammation exhibited, and the palpebral and bulbar mucosa are scored on the extent of chemosis, redness and discharge.

In this system of scoring such serious corneal lesions as pannus and phlyctenar reactions are not recorded. These reactions do not fully develop until the 7th day reading and persist for weeks. Any preparation eliciting such reactions is deemed to be too severe an irritant for consideration for cosmetic use about the eyes at that concentration.

A number of cationic, anionic and nonionic surface agents used in cosmetic preparations was studied for eye mucosa irritation. The "maximal tolerated concentrations"—as given in column 3 of Table 2 are not to be interpreted as the concentrations of these agents producing no eye reactions, but were the concentrations at which no corneal or iris lesions were evident by the seven-day reading.

Many surface active preparations contain less than 100

TABLE 2
Eye Irritation Studies With Surface Active Agents

Trade Name	Chemical Designation	Maximal Tolerated Concentrations of Active Ingredients
A. CATIONICS		
BTC®	Lauryldimethylbenzylammonium Chloride	0.5%
Hyamine 1622®	p-Disobutylphenoxymethyl-dimethylbenzylammonium Chloride	0.5%
Roccal®	Alkyldimethylbenzylammonium Chloride	0.5%
Isothane Q-15®	Laurylisoquinolinium Bromide	0.8%
Ethyl Cerab®	Cerylethyl-dimethylammonium Bromide	1.0%
Triton X-400®	Stearyldimethylbenzylammonium Chloride	1.0%
B. ANIONICS		
Nacconal NRSF®	Alkylaryl Sulfonate	5.0%
Miranol SM®	Capryl Imidazolane Derivative	10.0%
Aerosol OT®	Diactylester of Sodium Sulfosuccinic Acid	15.0%
Duponol WA®	Sodium Lauryl Sulfate	20.0%
Orvus WA®	Sodium Lauryl Sulfate	20.0%
Triton X-200®	Sodium salt of Alkylated Aryl polyether Sulfonate	28.0%
C. NONIONICS		
Triton X-100®	Alkylated Aryl Polyether Alcohol	5.0%
Nonic 218®	Polyethylene Glycol tere-dodecyl Thioether	10.0%
Alrasol C®	Fatty Acid Alkide Condensate	10.0%
Neutrolyx 600®	Aromatic Polyglycol Ether Condensate	15.0%
Detargem 1011®	Secondary Amide of Lauric Acid	15.0%
Ninol 2012®	Fatty acid Alkanolamine Condensate	20.0%
Span 20®	Sorbitan Monolaurate	100.0%
Tween 20®	Sorbitan Monolaurate	100.0%
Span 80®	Polyoxyethylene Derivative	100.0%
Tween 80®	Sorbitan Monooleate	100.0%
Arlacel A.®	Polyoxyethylene Derivative	100.0%
	Mannide Monooleate	100.0%



FIGURE 1

Normal eye mucosa of the albino rabbit. (Purpose of the slide is to show the normal cornea, iris and conjunctivae (no treatment).)

per cent active ingredient. All data reported in Table 2 are calculated on the basis of active ingredient. Dilutions, where necessary, were made with distilled water.

Surface active agents penetrate the eye mucosa readily to produce lesions. The cationics included in our studies produced severe lesions at relatively low concentrations. The nonionics, generally, were the least toxic but these and the anionics do exhibit marked differences in toxicity among their individual members. As reported in Table 2 the maximal tolerated concentration for sodium lauryl sulfate is 20 per cent. However, other lots of the same agent have varied from 15 to 25 per cent as the maximal tolerated concentrations for the eye mucosa.

The final formulation of a cosmetic will include other substances, such as solvents, vehicle, preservatives, etc., and often a combination of surface active agents. Such combinations in final formulation may produce eye irritation in excess of that anticipated from a simple addition of the irritant potential-



FIGURE 2

Picture made 3 days after instillation of 0.1 cc of a ten per cent water solution of a common household soap. Cornea and iris normal, slight congestion of blood vessels of conjunctivae. (This is considered an insignificant reaction).

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FIGURE 3

Picture taken 3 days after instillation of 0.1 cc of a Bubble Bath preparation consisting of 96% nonionic surface active agent. Illustrates an acute severe reaction—almost complete opacity of entire cornea, severe iritis, severe chemosis and redness of conjunctivae, moderate purulent discharge.

ties of the component parts. An example to illustrate this combination of irritant effects was encountered in these studies by simply combining solutions of a cationic with a nonionic agent. The corneal and iris lesions produced by either a 20 per cent alkyl aryl polyether alcohol (nonionic) or 1 per cent lauryl isoguanidine bromide (cationic) require approximately 4 weeks to heal. A solution containing both of these agents in these concentrations will produce eye lesions which have not cleared in 10 months of observation.

Summary Conclusions

1. Surface active agents penetrate the eye mucosa freely to produce lesions.
2. In some instances variations in local toxicity to eye mucosa were observed between different lots of the same preparation.



FIGURE 4

Picture taken 105 days after instillation of 0.1 cc of a shampoo preparation containing 18% nonionic agent with 2% of a cationic agent. Illustrates permanent or semi permanent eye injury, connective tissue scarring of cornea, with invasion of the cornea by blood vessels—the aftermath of severe pannus, and a chronic conjunctivitis.

The Toller Goods Association
Number 17 • May, 1952

3. The introduction of surface active agents in a preparation may produce irritation in excess of that which might be anticipated by the simple addition of the irritant effects of the individual agents.
4. Wide variations in toxicity were observed in the anionic and nonionic groups. The order of toxicity was cationic > anionic > nonionic.
5. A cosmetic formulation containing a surface active agent should be tested for eye irritant properties if such a preparation may gain access to the eye mucosa during normal use or from such use as is customary or usual.

Discussion

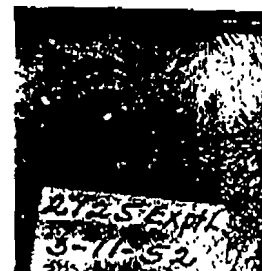
MR. DE NAVARRE: Dr. Draize, I don't think you made it quite clear whether those percentages you showed were of the surface-active agent as sold or was that 100 per cent active material?

DR. DRAIZE: Surface active agent preparations on the market may vary considerably in percentage of active ingredient. In the data reported, dilutions when necessary, were made on the basis of the declared active ingredient content.

DR. LAUFFER: I would like to be sure that I understood your remarks on the range of irritation of sodium lauryl



4A



4B

FIGURE 4A

Picture taken 8 days after instillation of 0.1 cc of a shampoo preparation containing 23% nonionic and 0.2% cationic surface active agent. Illustrates with the preceding slide the serious additive effects obtained by combination of agents. This slide is an example of severe early pannus, plus other reactions such as moderate opacity of cornea, severe iritis, moderate chemosis and redness of conjunctivae with moderate discharge.

FIGURE 4B

Picture taken 15 days after instillation of 0.1 cc of a dandruff treatment shampoo containing 2% cationic surface active agent. Illustrates a severe phlyctenar reaction in the cornea, plus a slight diffuse corneal opacity, slight iritis and slight redness of conjunctivae with slight discharge.

sulfate. I believe you said it varied with the tolerated range, that it varied from 15 to 25 per cent. Was that variation due entirely to differences in material, or was there some variation caused by the limits of experimental accuracy, or was there a difference between two brands of the material?

DR. DRAIZE: Sodium lauryl sulfate under various brand names was tested. Differences in toxicity were noted not only among various brands but even among lots of the same brand. The impression was obtained that variation in toxicity could be obtained among lots of supposedly identical preparations.

DR. LAUFFER: There was then more variation than could be explained on the basis of animal variation?

DR. DRAIZE: A sufficient number of animals are used to make the results conclusive.

DR. LAUFFER: You could not correlate that difference with any known impurity?

Black and white photographs from color positives by W. Fairclay Mann.

DR. DRAIZE: Impurities in the preparations were not investigated. The preparations as submitted, or in some cases diluted with distilled water, have been reported.

MR. LATVEN: We have investigated between 45 and 50 surface-active agents following instillation in the eye and found essentially the same result.

However, we have got to add one thing, namely, that the nonionics can be just as irritating as the cationics in specific instances, as you have already pointed out. The incidence, however, is less. We find around 25 per cent of all nonionics are irritating in consideration of corneal opacity and around 62 per cent of anionics fall into the opacification class, where it is 100 per cent with the cationics.

As you have also pointed out, the important question is whether or not the final formulas produce corneal opacity. That raises a very important question, namely: When one investigates a final formulation and obtains results such as that, only one out of nine or ten animals shows opacification. How can one interpret it? I must admit complete ignorance.

DR. DRAIZE: Occasionally one serious reaction only is obtained in a group of test subjects. Such a single reaction is deemed significant, since in the general population an occasional sensitive individual may be encountered, and from a standpoint of overall safety such an individual may not be overlooked.

MR. LATVEN: I wonder if I could ask another question on your interpretation, namely, the insidious character of a number of these surface-active agents is the fact that they don't produce pain on instillation. There is no blepharospasm in the animal, quite unlike that produced by soap, castile or tincture of green, either one. In instances where the incidence of corneal opacity is relatively low, that is, let's say, ten per cent of the population of animals, and there is no sense of pain on instillation, which is not so good, do you think it

would be wise to add in such a formulation an irritant which does not produce corneal opacity?

DR. DRAIZE: It is true that some surface active agent preparations seem to exert local anesthetic properties, which is unfortunate, since such a preparation may cause considerable damage before the subject becomes aware of injury. The majority of surface active agents elicit varying degrees of pain upon instillation in the eye, which in a sense is desirable since it makes the subject aware of the necessity for immediate treatment.

It would be unwise, however, to add another irritant for the sole purpose of eliciting pain to warn of potential harm.

DR. RUSSELL: I have two questions about the scoring. You have three groups of three rabbits in each group. For your final score do you average those three?

DR. DRAIZE: The scores are not averaged.

DR. RUSSELL: You have such-and-such a score for those that are added undiluted and not rinsed out, then another score for those rinsed out of the eyes. Do you get very much the same result, whether you rinse them or not?

DR. DRAIZE: With some preparations rinsing makes a considerable difference. As mentioned in the body of the paper, rinsing may actually be detrimental; not often, but it sometimes is. The scores are not added or averaged. Absolute clarity of the cornea and iris in all three rabbits in all categories must be obtained by the seventh day.

DR. RUSSELL: Rinsed or unrinsed?

DR. DRAIZE: Rinsed or unrinsed. That is right.

DR. RUSSELL: Is the maximum tolerated dose scored on the basis of clearing in under seven days?

DR. DRAIZE: That is right, between the fourth and the seventh day.

BIBLIOGRAPHY

1. Draize, John H., G. Woodward & H. O. Calvery, *J. Pharmacol. Exptl. Therap.* 84, 871-90 (Dec. 1944).

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REACTIVITY OF THE OCULAR TISSUES TO WETTING AGENTS*

KENNETH C. SWAN, M.D.
Iowa City, Iowa

Many highly surface-active compounds have been synthesized recently. Even in minute concentrations they lower the surface tension of water and thereby facilitate miscibility of water with water-insoluble substances; that is, wetting. These wetting agents are widely used as substitutes for soap, but also have other properties of importance in ophthalmology. Several are strong antiseptics with high bactericidal power and reportedly low tissue-toxicity;^{1,2} consequently, they have been used in treatment of external ocular infections.³ The combination of detergent and bactericidal action has made them particularly useful in preoperative preparation of the conjunctiva and the skin of the lids, and in sterilization of delicate ophthalmic instruments. Also, aqueous solutions of wetting agents are excellent vehicles for ophthalmic drugs in that they inhibit bacterial and fungal growth and facilitate absorption⁴ of certain drugs; for example, carbamylcholine chloride.¹ These agents are also used to stabilize emulsions and suspensions of other ophthalmic drugs; for example, sulfathiazole.⁵ Recently, a new class of cycloplegic and mydriatic drugs with the properties of wetting agents has been synthesized by the writer and Norman G. White.⁶ These new compounds are surface-active esters of choline, and diethyl-aminoethanol.

* From the Department of Ophthalmology, College of Medicine, State University of Iowa. Part of a study being conducted under a grant from the John and Mary R. Markle Foundation.

¹ The facilitation of drug absorption is probably due to increased permeability of the surface epithelium and improved contact between the drug solution and the epithelium.

Considering the numerous applications of wetting agents to ophthalmic therapeutics and the increasing liability of accidental ocular inoculation in the home and industry, investigation of the possible toxic effects of wetting agents on the ocular tissues was considered important. O'Brien and Swan⁷ reported that 0.04- to 0.05-percent solutions of zephiran chloride produced superficial punctate disturbances of the epithelium. Otherwise, the effects of wetting agents have received little attention as factors contributing to irritation and injury either from accidental inoculation or after instillation of drug solutions into the conjunctival sac. In fact, manufacturers of wetting agents have stressed their low tissue toxicity.²

Over a period of five years, wetting agents were administered by the author to hundreds of patients in the Eye Clinic of the State University of Iowa. Many of these patients received only single instillations of the drugs, whereas a few have received instillations two to four times daily for as long as three years. Tolerance to the drug solutions was determined by repeated examinations of the conjunctiva and cornea by slitlamp biomicroscopy. In addition, patients were questioned regarding irritation. The studies were made with zephiran, dipical, phemerol, aerosols, and several other wetting agents, including the new class of short-acting cycloplegic and mydriatic drugs.

Strong solutions of wetting agents—that is, 0.1-percent zephiran chloride—produced a characteristic conjunctival reaction. Following instillations of a single drop (0.50 c.c.) into the conjunctival sac

of human volunteers, hyperemia and edema of the conjunctiva developed. Lacrimation was profuse. Slitlamp examination revealed thickening and decreased transparency of the superficial layers of the conjunctiva associated with

the desquamated epithelium often became rolled into strands of sufficient size and consistence to create a foreign-body sensation.

Characteristic corneal lesions occurred also. Within 90 seconds after instillation

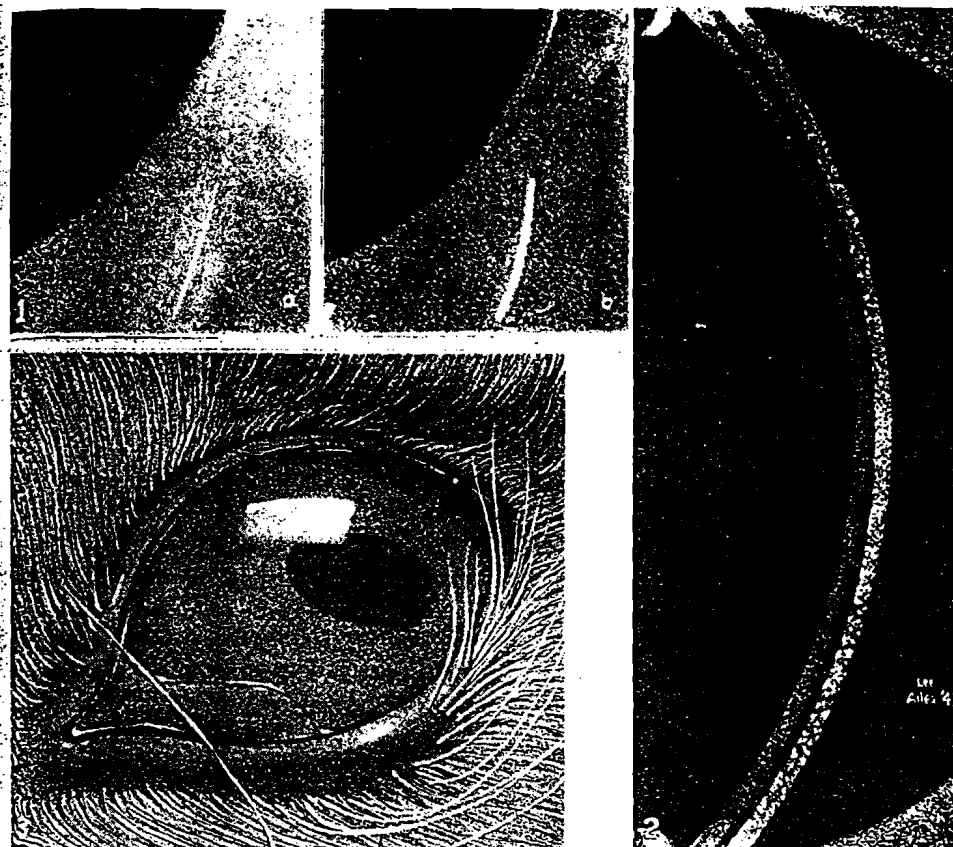


Fig. 1 (Swan). Biomicroscopic appearance of normal conjunctiva: a, after instillation of 0.1-percent zephiran; b, the conjunctiva is edematous and hyperemic.

Fig. 2. Superficial punctate lesions of the corneal epithelium produced by wetting agents.

Fig. 3. Tremendous edema of rabbit's cornea 14 hours after injection of a wetting agent into the anterior chamber.

marked dilatation of the superficial capillaries (fig. 1). Engorgement of the deeper and larger conjunctival vessels followed in some individuals. Frequently, actual desquamation of the conjunctival epithelium occurred either as pseudomembranes or as strands or clumps of clear, tenacious discharge. In the lower fornix,

of a drop of 0.1-percent zephiran chloride, multiple, punctate, gray areas in the corneal epithelium were evident with the aid of the biomicroscope (fig. 2). These tiny areas gradually became confluent, and within 10 minutes could be seen with the naked eye as a gray haze in the corneal surfaces. Roughening and drying of the

epithelium, such as is noted after instillations of topical anesthetics, did not occur. A similar but much less severe disturbance of the corneal epithelium was seen in the corneas of several patients who had been instilling 0.03- to 0.04-percent zephiran solution into their conjunctival sacs three to four times daily over periods of from two to eight weeks. These patients complained of the sensation of sand in their eyes. Recovery was rapid in all cases. In most, the conjunctiva and cornea returned to normal within 12 hours.

Individuals varied in their sensitivity to solutions of wetting agents; however, no evidence of idiosyncrasy was observed nor total lack of reactivity in individual patients. There was, moreover, some difference in the intensity and duration of irritation produced by the different wetting agents, even in solutions having the same air-water interfacial tension. The surface tension of solutions is not directly proportionate to the concentration of the wetting agent; therefore, dilution by tear and conjunctival secretion does not have an equal effect on the surface activity of all wetting agents. However, determination of the air-water interfacial tension provided some index as to whether a solution containing a surface-active compound would be irritating. When the pH of the solution was neutral (pH 6.5 to 7.5) and the solution isotonic,* concentrations of wetting agents which lowered the surface tension at air-water interface to less than 37 dynes per centimeter at 25 degrees and which maintained a surface tension below 40 dynes despite dilution of several times, were consistently described as irritating although objective changes could not always be observed.

The effects of wetting agents seemed

* The hydrogen-ion concentrations of the various solutions were determined with a glass electrode in all instances. Osmotic-pressure measurements were made by the vapor-pressure method.

cumulative. A single instillation was often well tolerated whereas a second or third instillation at short intervals produced discomfort and objective signs of irritation. A number of external inflammations was observed to be aggravated by too frequent instillations of wetting agents administered as bactericidal agents. It is important, therefore, to limit the frequency of administration of wetting agents to a few instillations daily until the patient's tolerance is established.

The injurious effects of solutions of wetting agents instilled into the conjunctival sac seemed limited to the most superficial layers of the cornea and conjunctiva, even when repeated instillations were made. One-tenth-percent solutions of phemerol and zephiran were instilled into the conjunctival sacs of rabbit eyes two to three times daily for periods of one to three months. The corneal epithelium became thickened, rough, and superficial vascularization developed, but no damage to the deeper layers of the cornea or to the intraocular tissues was noted by either slitlamp biomicroscopy or microscopic study of stained sections. Even when undissolved aerosol OT was placed directly on the cornea, the inflammatory reaction was limited to the superficial layers.

The skin of the lids was less readily irritated by wetting agents than was the conjunctiva, but even so was more sensitive than skin elsewhere on the body. Concentrations advocated by the manufacturers for use on the skin occasionally produced irritation of the lids.

It was considered desirable to investigate the effects of wetting agents on the intraocular tissues for several reasons. It was thought that because of their advertised low tissue-toxicity and high bactericidal action, wetting agents could possibly be added to solutions used for irrigation of the anterior chamber to lessen

the danger of postoperative infections in ophthalmic surgery or to treat anterior-segment infections. Also, accidental intraocular inoculation of wetting agents might occur in industry or in instances where solutions of these agents were carelessly used to prepare the conjunctival sac or sterilize instruments for intraocular surgery.

To determine the influence of wetting agents on the intraocular tissues, the anterior chambers of albino-rabbit eyes were irrigated with 0.68-percent sodium chloride containing known concentrations of wetting agents. Limbic punctures were made with two, sharp 27-gauge needles so joined by a syringe system that as aqueous was withdrawn into one syringe an equal quantity of the test solution was injected from the other. By this technique it was possible to control the concentration of the wetting agent in the anterior chamber without incurring the reaction which follows emptying of the anterior chamber or gross alterations in intraocular pressure. Slitlamp studies when sodium fluorescein was added either to the test solution or injected intravenously indicated that the test solutions permeated to all parts of the anterior chamber and replaced most of the normal aqueous.

Control injections made with 0.68-percent sodium chloride were found to create a mild iridocyclitis, but after 24 hours there was no evidence of inflammation provided the iris had not been touched by the needles. In contrast, minute concentrations of wetting agents—for example, 0.025- to 0.050-percent zephiran—resulted in a violent reaction in the rabbit eye. Slitlamp biomicroscopy revealed a swollen, gray corneal endothelium and in some instances vesicles were observed to form and rupture, leaving Descemet's membrane exposed. There were marked engorgement of the iris vessels, edema of the iris stroma, and a profuse outpouring

of fibrin and protein into the anterior chamber. The suture markings on the aqueous surface of the lens became prominent, but no definite lens opacities developed. Within 12 hours, the corneal stroma became edematous and the epithelium bedewed (fig. 3). The acute inflammatory process in the iris usually subsided over a period of a few days to a week, although in several instances degeneration or secondary glaucoma followed. The cornea seldom returned to normal and in many instances became opaque and vascularized. The reaction in the intraocular tissues appeared proportionate to the concentration of the wetting agent in the anterior chamber, for example, concentrations of 0.05-percent zephiran or phemerol were almost always followed by serious sequelae whereas the eye generally recovered from concentrations of 0.01 percent.

DISCUSSION

The minimal concentrations of wetting agents producing irritation in the conjunctival sac are greater than those that are effective therapeutically. For example, Allen⁸ found that concentrations of 0.002-percent zephiran inhibit the respiration of hemolytic staphylococci of known ocular pathogenicity* but concentrations as high as 0.025 percent were tolerated in the conjunctival sac. The concentrations necessary to facilitate absorption of drugs from the conjunctival sac or added to ointments or emulsions to produce stability and smooth preparations are also less than those producing irritation with a single administration. Most of the surface-active mydriatic and cycloplegic drugs developed by Swan and White are generally not irritating in therapeutic doses. In fact, one of these

* In combination with inorganic mercurials, the minimal effective bactericidal concentration of zephiran was found to be even more dilute.

new drugs, dibutoline, has been proved effective in allaying anterior-segment inflammations.⁹

SUMMARY AND CONCLUSIONS

Although low tissue-toxicity of wetting agents has been stressed in the literature and commercial advertisements, these surface-active compounds are capable of producing reactions in the ocular tissues. Surface activity must therefore be added to variations in pH and osmotic pressure as possible reasons for irritation from ophthalmic medicants. The surface tension (air-water interface) of a given drug solution provides some evidence as to whether a wetting agent is present in sufficient concentration to produce irritation in the conjunctival sac.

In the conjunctiva and corneal epithelium, wetting agents produce characteristic reactions that are greatly increased by

frequent or prolonged administration. Although superficial and transitory, these reactions are distressing to patients. Fortunately, the concentrations producing irritation in the conjunctival sac are usually greater than the minimal effective therapeutic concentrations, provided the solutions are not administered too frequently.

Wetting agents injected into the anterior chamber in minute concentrations produce violent reactions. The endothelium of the cornea seems particularly susceptible to damage. Care must be taken to avoid accidental intraocular introduction when these agents are used in sterilization of instruments for intraocular surgery, in preoperative preparation of the conjunctival sac, or in treatment of penetrating wounds.

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REFERENCES

- ¹ Welch, H., and Brewer, C. M. The toxicity indices of some basic antiseptic substances. *Jour. Immunol.*, 1942, v. 43, pp. 25-30.
- ² Zephiran-Biochem. number 641-A-258. Alba Pharmaceutical Co., 1942, New York.
- ³ Smith, C. S. Some recent advances in ophthalmology. *Jour. Missouri State Med. Assoc.*, 1939, v. 36, p. 72.
- ⁴ Swan, K. C. Advances in the medical treatment of glaucoma. *Jour. Lancet*, 1942, v. 62, p. 7.
- ⁵ Bellows, J. G., and Guttman, M. Application of wetting agents in ophthalmology. *Arch. Ophth.*, 1943, v. 30, pp. 352-357.
- ⁶ Swan, K. C., and White, N. G. Some new choline esters with cycloplegic and mydriatic properties. *Proc. Soc. Exper. Biol. and Med.*, 1943, v. 53, pp. 164-166.
- ⁷ O'Brien, C. S., and Swan, K. C. Doryl in the treatment of glaucoma simplex. *Trans. Amer. Ophth. Soc.*, 1941.
- ⁸ Allen, I. H. Personal communication.
- ⁹ Swan, K. C., and White, N. G. Di-N-Butylcarbaminoylecholine sulfate: A new cycloplegic and mydriatic drug. *Arch. of Ophth.*, 1944, v. 31, April, p. 289. See also *Amer. Jour. Ophth.*, 1944, v. 27, p. 933.

Hyamine 1622 Eye Irritation Scores

Hyamine 1622, 50% Concentration (% Active)	Summary of Mean Ocular Scores (Standard Draize Test)																			
	Time After Test Material Administration																			
	1-Hour				Day 1				Day 2				Day 3				Day 4			
	Cornea	Iris	Conj	Total	Cornea	Iris	Conj	Total	Cornea	Iris	Conj	Total	Cornea	Iris	Conj	Total	Cornea	Iris	Conj	Total
1.0	0.0	4.2	9.3	13.5	0.0	2.5	10.7	13.2	0.0	0.0	9.3	9.3	0.0	0.0	7.7	7.7	0.0	0.0	4.0	4.0
1.6	1.7	5.0	8.3	15.0	5.8	2.5	9.7	18.0	8.3	0.8	8.0	18.8	4.2	0.0	6.3	10.5	0.0	0.0	4.7	4.7
3.2	0.8	5.0	10.3	16.2	13.3	4.2	10.0	27.5	12.5	4.2	10.3	27.0	12.5	4.2	9.3	26.0	9.2	3.3	8.7	21.2
	Day 7				Day 10				Day 14				Day 18				Day 21			
	Cornea	Iris	Conj	Total	Cornea	Iris	Conj	Total	Cornea	Iris	Conj	Total	Cornea	Iris	Conj	Total	Cornea	Iris	Conj	Total
1.0	0.0	0.0	0.7	0.7 (2)	0.0	0.0	0.7	0.7	0.0	0.0	0.0	0.0	0.0	0.0	1.0	1.0 (1)	0.0	0.0	0.7	0.7 (1)
1.6	0.0	0.0	2.7	2.7 (4)	0.0	0.0	1.7	1.7 (3)	0.0	0.0	0.7	0.7 (1)	0.0	0.0	1.0	1.0 (1)	0.0	0.0	0.7	0.7 (1)
3.2	13.3	1.7	5.7	20.7	15.0	0.0	4.6	19.7 (5)	26.7	0.0	2.0	28.7 (5)	20.0	0.0	1.3	21.3 (4)	20.0	0.0	1.3	21.3 (4)

Key: Conj = Conjunctiva

Note: Numbers in parentheses indicate number of animals showing positive response when n is not 6.

Hyamine 1622 Eye Irritation Scores

Summary of Mean Ocular Scores (Standard Draize Test)																					
Hyamine 1622, 50% Concentration (% Active)		Time After Test Material Administration																			
		1-Hour				Day 1				Day 2				Day 3				Day 4			
		Cornea	Iris	Conj	Total	Cornea	Iris	Conj	Total	Cornea	Iris	Conj	Total	Cornea	Iris	Conj	Total	Cornea	Iris	Conj	Total
1.0		0.0	4.2	9.3	13.5	0.0	2.5	10.7	13.2	0.0	0.0	9.3	9.3	0.0	0.0	7.7	7.7	0.0	0.0	4.0	4.0
1.6		1.7	5.0	8.3	15.0	5.8	2.5	9.7	18.0	8.3	0.8	8.0	18.8	4.2	0.0	6.3	10.5	0.0	0.0	4.7	4.7
3.2		0.8	5.0	10.3	16.2	13.3	4.2	10.0	27.5	12.5	4.2	10.3	27.0	12.5	4.2	9.3	26.0	9.2	3.3	8.7	21.2
		Day 7				Day 10				Day 14				Day 18				Day 21			
		Cornea	Iris	Conj	Total	Cornea	Iris	Conj	Total	Cornea	Iris	Conj	Total	Cornea	Iris	Conj	Total	Cornea	Iris	Conj	Total
1.0		0.0	0.0	0.7	0.7 (2)	0.0	0.0	0.7	0.7	0.0	0.0	0.0	0.0								
1.6		0.0	0.0	2.7	2.7 (4)	0.0	0.0	1.7	1.7 (3)	0.0	0.0	0.7	0.7 (1)	0.0	0.0	1.0	1.0 (1)	0.0	0.0	0.7	0.7 (1)
3.2		13.3	1.7	5.7	20.7	15.0	0.0	4.6	19.7 (5)	26.7	0.0	2.0	28.7 (5)	20.0	0.0	1.3	21.3 (4)	20.0	0.0	1.3	21.3 (4)

Key: Conj = Conjunctiva

Note: Numbers in parentheses indicate number of animals showing positive response when n is not 6.

Magnusson-Kligman Maximization Test With Benzethonium Chloride in Guinea Pigs

General Information

Reference: Draft Final Report to Lonza Inc.
Report Date: September 1995
Testing Laboratory: Product Safety Labs

Study Design

Test System: Female Hartley guinea pigs
Age at Start of Test: Young adult
Test Substance Concentrations: Induction: 2% aqueous solution for intradermal injection and topical application. A 2% aqueous solution of benzethonium chloride is the minimum irritating concentration in this test system.
Challenge: 1% aqueous solution (maximum nonirritating concentration).

Treatment Regimen: Induction:
i. 0.1 ml intradermal injections with the test substance alone, the test substance in Complete Freund's Adjuvant, or Complete Freund's Adjuvant alone;
ii. 0.5 ml topical application of the test substance one week after the intradermal injection. Topical application sites were occluded for 48 hours;
Challenge: 0.5 ml topical application of the test substance to pretreated and naive animals two weeks after the topical induction application. The application sites were occluded for 24 hours.

Positive Control: Dinitrochlorobenzene. The treatment regimen and experimental evaluations were performed as described for the test substance.

Number of Animals per Group: Test group: 20 animals for induction and challenge; 10 naive animals for challenge;

Positive control: 10 animals for induction and challenge; 5 naive animals for challenge.

Experimental Evaluation: Evaluation of skin sites 48 hours after application of the test substance.

Results and Conclusion

The test substance did not produce a delayed contact skin sensitization reaction in any of the animals tested. A positive delayed contact sensitization response observed for animals treated with dinitrochlorobenzene demonstrated the sensitivity of this test system. Under the conditions of this study, benzethonium chloride is not a delayed contact sensitizer.

P.B.

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NTP REPORT ON THE
IMMUNOTOXICITY OF BENZETHONIUM CHLORIDE
 (CAS No. 121-54-0)
 IN FEMALE B6C3F1 MICE
 (CONTACT HYPERSENSITIVITY STUDIES)

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Introduction: Benzethonium chloride is used as an antiinfective, in veterinary medicine as a topical antiseptic, and as a cationic detergent. Cationic detergents are used agriculturally in herbicides and in antiseptics, spermicides, astringents, germicides, disinfectants, and preservatives. Hypersensitivity reactions have been reported in 12 of 42 individuals treated with a topical preparation containing benzethonium chloride (*Acta Otolaryngol.* 100: 414, 1985).

Design: Benzethonium chloride (lot # W0061/01) was obtained from Rohm and Haas, Inc. (Philadelphia, PA) and was > 98% pure as determined by HPLC. The material was prepared in 95% ethanol, which also served as the vehicle. A 0.5% solution of 1-fluoro-2,4-dinitrobenzene (DNFB; Sigma Chemical Corp., Lot No. 87F-3777; > 99.0% pure as determined by HPLC) was used as the positive control. In the primary irritancy studies, a 10% concentration of benzethonium chloride was not significantly irritating, therefore, 20% was chosen as the challenge concentration. For the hypersensitivity test, B6C3F1 mice were divided into 7 treatment groups of 8 mice/group and administered the test compound at the concentrations shown in Table 1. The irritancy response was determined by monitoring the extravasation of ¹²⁵I-bovine serum albumin into the testing area. The contact hypersensitivity response was determined by monitoring the infiltration of ¹²⁵I-iododeoxyuridine labeled cells into the challenge site.

Table 1. Study Design: Contact Hypersensitivity Study with Benzethonium Chloride

Group (n)	Description	Sensitization	Challenge
1 (8)	Vehicle	Vehicle	Vehicle
2 (8)	Baseline Control	Vehicle	20% BZ
3 (8)	Experimental	1% BZ	20% BZ
4 (8)	Experimental	3% BZ	20% BZ
5 (8)	Experimental	10% BZ	20% BZ
6 (8)	DNFB Positive Control	0.5% DNFB	0.5% DNFB
7 (8)	DNFB Negative Control	Vehicle	0.5% DNFB

BZ = benzethonium chloride

DNFB = 1-fluoro-2,4-dinitrobenzene

Results: There were no treatment-related effects on survival or body weights. The Hypersensitivity Index (HI) values obtained with benzethonium chloride sensitization at 1%, 3%, and 10% were not significantly different from the baseline control value (Figure 1). The positive control group (6) produced a statistically significant hypersensitivity response at a sensitizing and challenge concentration of 0.5% DNFB.

Conclusions: Under these experimental conditions, no statistically significant group or dose-dependent contact hypersensitivity responses to benzethonium chloride were observed in mice by dermal exposure.

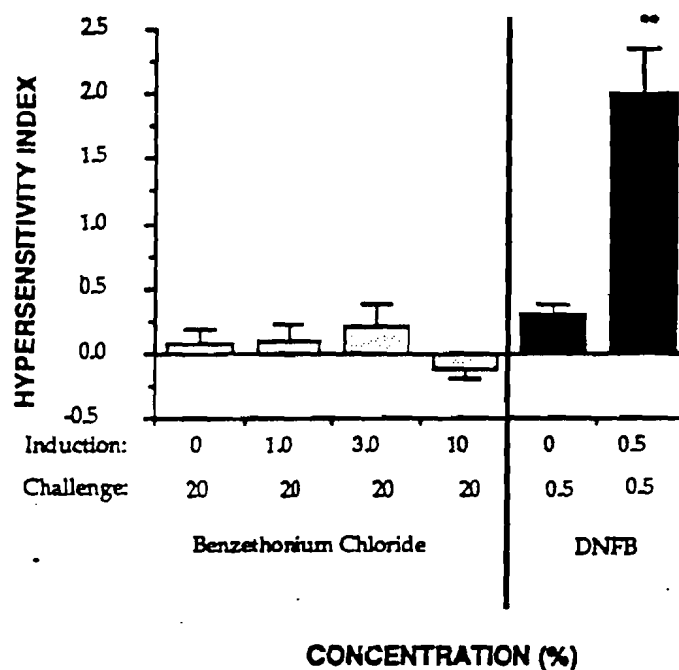


Figure 1: Contact Hypersensitivity Response to Benzethonium Chloride in Female B6C3F1 Mice: Mice were divided into 7 groups of 8 mice/group and treated as described in Table 1 (Group 1 not shown). The Hypersensitivity Index (HI) was calculated as described in Appendix B. Results are shown as the mean \pm SE; ** $P < 0.01$ vs 0% control

Rat Maternal and Fetal Absorption of

^{14}C -Benzethonium Chloride (^{14}C -BTC)

Prepared by:

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February 23, 1977

Rat Maternal and Fetal Absorption of

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SUMMARY

- ^{14}C -Benzethonium Chloride was detected in maternal blood and urine of pregnant rats who received orally 1.125 mg/kg/day and 3.558 mg/kg/day on days-6 through 15 of gestation. Maximum blood levels of 1.5 and 0.97 ng/g of ^{14}C -BTC were obtained after cumulative dosing to day-15 and these values were reduced to 0.08 and 0.34 ng/g respectively by day-18 (three days after the last treatment).
- In the urine the amount of ^{14}C -BTC detected was also in nanograms: the greatest amount, 52 and 140 ng/ml, appeared after a single dose which by day-18 decreased to 1.0 and 5.0 ng/ml respectively.
- In some fetuses nanogram amounts of ^{14}C -BTC were detected. Fetal absorption was erratic and varied from non-detectable in some pups to above background in other pups from the same litter. In the rats dosed with 1.125 mg/kg/day; 2.47 ng/g fetus were recovered on day-18 while none was detectable in the day-6 and 15 samples. In the high dose group (3.558 mg/kg/day) 1.34 ng/g was detected only in the pups from day-15.
- Virtually, all the administered radioactivity was recovered in the maternal feces and carcass after the initial dose. On day-18, three days after the last treatment, less than 8% of the ^{14}C -BTC radioactivity was eliminated in the feces or remained in the carcass.

I. Introduction

The study was designed to assess if ^{14}C -Benzethonium Chloride (^{14}C -BTC) when administered to rats by gastric intubation was absorbed into the maternal circulation and transported into and detectable in the fetus. Dose levels of 1.125 and 3.558 mg ^{14}C -BTC/kg/day were administered on days-6 through 15 of gestation. The doses selected were chosen on the basis of a Segment II Rat Teratology Study (Project No. 75-1495A) which was performed to evaluate the safety of Benzethonium Chloride.

The data contained within this report are preliminary.

II. Materials

A. ^{14}C -Benzethonium Chloride, (^{14}C -BTC)

The material was prepared by New England Nuclear* as a custom synthesis (Carbon-14) compound labeled on the benzyl methylene group: Lot #922-149, 5.2 mCi, 2.27×10^7 dpm/mg, 506 mg.

Thin layer chromatography (TLC) using two different solvent systems and autoradiography showed that the radioactive purity of the ^{14}C -BTC was greater than 99%.

Identity and chemical purity were checked by a separate TLC which compared:

- a. fresh Hyamine 1622 (Rohm & Haas' BTC)
- b. a mixture of above and ^{14}C -BTC
- c. ^{14}C -BTC

The spots were disclosed for identification by spraying with Evans Blue. The results showed only one spot of equal Rf for a, b and c. Since sample b, the mixture, only exhibited one spot, the chemical purity and identity of the ^{14}C -BTC was established.

1. One Percent Stock Solution

All of the ^{14}C -BTC was made up to 50.6 cc with 200 proof USP ethanol to make a 1% (w/v) solution equivalent to a 1.24% (w/w) solution.

2. Low Level Dosing Solution 0.1125 mg/cc

5.625 cc of the stock solution was diluted to 500 cc with water. The specific activity of the solution was found to be 2.57×10^6 dpm/cc.

3. High Level Dosing Solution 0.3558 mg/cc

17.8 cc of the stock solution was diluted to 500 cc with water with a resulting specific activity of 8.05×10^5 dpm/cc.

* 575 Albany Street, Boston, MA 02118

B. Non-Radioactive Benzethonium Chloride 0.112 mg/cc

Low Level Dosing Solution:

56.0 mg of Hyamine 1622 (Rohm & Haas) was diluted to 500 cc with water.

III. Test Animals

Forty-five Long-Evans sexually mature female rats were obtained from Bio/dynamics Laboratory, Inc. They were from the same population used in a Segment II Teratology Study (Project No. 75-1495A) performed at Bio/dynamics Laboratory, Inc. and reported on December 2, 1976. The rats were obtained on day-2 of pregnancy and the specific details of the husbandry are detailed in that report (Project No. 75-1495A).

IV. Experimental Design

A. Deployment of Rats - Randomly assigned as follows:

<u>Test Day</u>	<u>¹⁴C-BTC Levels, mg/kg/day</u>		
	<u>1.125</u>	<u>3.558</u>	<u>1.125 (Control - Cold BTC)</u>
6	5+	5	5
15	5	5	5
18	5	5	5

+ number of rats/group

The rats were housed individually and provided fresh tap water and ground Purina Laboratory Chow ad libitum.

B. Dosing

All rats were dosed with a constant volume of 1.0 ml/100 g body weight throughout the study.

Day-6 Five rats from each group were intubated into the stomach with the respective dosing solutions. Immediately after dosing, each rat was placed for six hours in an Econo-Cage #110 Metabolism Unit (Maryland Plastics, Inc., New York, NY) and received ground Chow ad lib. At the end of six hours the rats were lightly anesthetized with ether and 1.0 ml of blood was withdrawn by cardiac puncture (1.0 ml syringe), 21 gauge, 1 inch needle; needle and syringe were washed with heparin; 1000 USP units/ml (Sherwood Medical Laboratories, Inc., St. Louis, MO). Residual heparin solution was allowed to remain only in the needle. After blood sampling, the rats were immediately returned to their individual metabolism cages.

Twenty-four hours after the initial dose, the rats were sacrificed by cervical dislocation. The abdomen was laid open and the fallopian tubes

and uterus at the vaginal-cervical junction clamped off with Holstead mosquito forceps. The entire uterus without excess external fat and ovaries was removed, placed in a Petri dish and saved for analysis. The blood, urine, feces and remaining carcass were also saved for analysis.

Day-15

Blood samples from the five rats in each group which were dosed daily from day-6 through 15 were obtained by cardiac puncture six hours after the last injection. The necropsy with tissue selection procedures was similar to those given for day-6 above except that the individual fetuses were removed from the uterus and freed from the placental membranes.

Day-18

Blood samples were taken from the remaining five rats from each group, which had not been dosed since day-15. The rats were sacrificed and the individual fetuses were removed from the uterus and freed from the placental membranes, urine and feces samples which were collected for 24 hours prior to sacrifice were saved for analysis.

V. Analysis of Tissue Samples

General Procedure

Radioactivity was counted in a Packard #3375 Tri-Carb Liquid Scintillation Spectrometer (Packard Instruments Co., Inc., Downers Grove, IL). Counting efficiency was determined for those samples directly added to the cocktail (Oxifluor, New England Nuclear) by using the Tri-Carb automatic external standardization feature. For combusted samples the overall efficiency was estimated from spiked control tissue samples that were combusted along with the experimental samples. Tissues were kept frozen until sampled. All vials were counted to a minimum 2.5 percent standard deviation.

Individual Tissue Samples

The tissues were processed as follows:

Blood

About 0.5 g duplicate samples was combusted in the Oxymat Automatic Oxidizer (Intertechnique Instruments, Inc., Teledyne, Fairfield, NJ).

Urine

Duplicate 2 ml samples were added directly to liquid scintillation cocktail for counting.

Feces

Feces were lyophilized and 20 to 30 mg samples were taken for combustion.

Uterus (from day-6 pregnant rats)

Each entire uterus with fetuses was lyophilized and combusted.

Fetuses (from day-15 and day-18 pregnant rats)

Each entire fetus (separated from placenta) was lyophilized and combusted individually.

Placenta (from day-15 and day-18 pregnant rats)

The remaining placenta, after removal of fetuses, was lyophilized, ground to a coarse powder and 0.4 to 0.5 g samples combusted.

Carcass

After removal of uterus, the entire remaining carcass was homogenized with 800 cc methanol in a one gallon capacity Waring Blendor. The homogenate was filtered and 2 ml aliquot of the filtrate was added directly to the cocktail for counting. The insoluble portion of the rat homogenate was dried and several representative samples were combusted. Several samples of the filter paper were counted directly in the cocktail. The individual values for the filtrate, carcass and filter paper was summed to give total carcass recovery.

The lyophilizer condensate was measured for radioactivity to detect any likely volatile ^{14}C metabolites; no activity was found in the condensate.

VI. Results

A. Rat Tissue Samples

The individual data itemized in the Appendix are summarized in Table I. The average values are listed for the respective groups with the range of observed values within the parentheses.

B. TABLE I

- 11 -

Rat Maternal and Fetal Absorption of ^{14}C -Benzethonium Chloride: Data++ Summary

Tissue	^{14}C -BTC Activity	DOSE: 1.125 mg/kg/day			DOSE: 3.558 mg/kg/day		
		Day			Day		
		6	15	18	6	15	18
Blood	ng/g	0	1.5 (.54-4.7)	.08 (0-.22)	0.200 (0-.8)	0.97 (0.6-1.72)	0.34 (0-.93)
Urine	ng/ml	52.00 (25-103)	28.00 (5-77)	1.0 (0-3.0)	140.000 (92-201)	47.00 (18-89)	5.0 (2-13)
Fetus	ng/g**	---	0	2.47 (.03-6.8)	---	1.34 (0.5-4.8)	0
Uterus	ng/uterus***	.98 (0-2.53)	---	---	.95 (.68-1.59)	---	---
Placenta	ng/g	---	71.00 (71)*	0.8 (0.8)*	---	4.6 (0-6.6)+	1.2 (0.7-1.8)+
Feces	ug/g	67.2 (48.3-97.1)	56.9 (13.7-107.4)	5.68 (3.1-14.1)	275.33 (214.2-366.2)	220.74 (123.0-364.5)	16.64 (11.7-25.0)
Carcass	ug/g	0.496 (.079-1.395)	0.413 (.015-1.44)	0.034 (.002-.098)	0.936 (.591-1.797)	0.614 (.285-1.484)	0.032 (.003-.065)

* One rat

** Average for those fetuses exhibiting ^{14}C -activity

*** Combined tissues of the uterus, placenta and fetus

+ Average for those placentas exhibiting ^{14}C -activity

* Values are averages for the group; values in parenthesis denote observed range

C. Blood and Urine

1. Blood

The data in Table I show that ^{14}C -BTC was recovered in each dosage group with the exception of rats receiving the single dose of 1.125 mg/kg on day-6 of gestation. One rat from this group died after cardiac puncture and the autopsy revealed the cause of death was related to the blood sampling procedure.

The amount of ^{14}C detected in the blood increased after repeated dosing from "zero" levels to an average of 1.5 ng/g ^{14}C on day-15 in the 1.125 mg/kg/day group. One rat from this group died several hours after cardiac puncture and the autopsy revealed the cause of death was due to this procedure. By day-18, the blood level decreased to 0.08 ng/g.

Similarly, rats dosed with 3.558 mg/kg/day showed an increased blood concentration from 0.2 ng/g on day-6 to 0.97 ng/g on day-15 and by day-18 it was decreased to 0.34 ng/g.

2. Urine

^{14}C -BTC was detected in urine from rats of each treatment group at each time sampling interval and appeared to be dose-related. The highest level average activity--52 ng/ml and 140 ng/ml--was measured in those rats treated on day-6. On day-15, after cumulative dosing, the urinary values were only 28 and 47 ng/ml respectively to low and high dose levels. By day-18 the urine concentrations were 1.0 and 5.0 ng/ml respectively to low and high dose levels.

D. Uterine, Placental and Fetal Tissues

1. Day-6

^{14}C -activity--0.98 ng/uterus and 0.95 ng/uterus--were detected in dams dosed with 1.125 and 3.558 mg/kg/day respectively which does not appear dose-related.

2. Day-15

2.1 Fetal Tissue

No ^{14}C -activity was detected in any of the fetuses from the dams dosed with 1.125 mg/kg/day. Small

amounts (average 1.34 ng/g) of ^{14}C -activity were detected in the fetal tissue from the dams dosed with 3.558 mg/kg/day. The average values listed in Table I represent the mean values only for those fetuses exhibiting ^{14}C -activity. The frequency with which activity appeared in the fetus varied with the respective litters. For example, only 4/17 pups from one dam showed activity over background while 14/16 pups showed activity from another dam. No radioactivity was detected in 11/11 pups from another dam in the same dosage group. Transport from the maternal circulation into the fetal tissues appeared random and inconsistent. Because of the extremely low levels detected i.e., ng/g, and the apparent randomness of the distribution within a given litter, the significance of these data in terms of safety is inconclusive.

2.2 Placental Tissue

Only one rat from the group dosed with 1.125 mg/kg/day exhibited ^{14}C -activity (71 ng/g) in the placental tissue while 2/5 exhibited placental ^{14}C -activity (4.6 ng/g) in the group dosed with 3.558 mg/kg/day.

3. Day-18

3.1 Fetal Tissue

The amount of detectable ^{14}C -activity was an average of 2.47 ng/g for fetuses of dams receiving 1.125 mg/kg/day, whereas, no ^{14}C -activity was detected in fetuses from dams receiving 3.558 mg/kg/day.

3.2 Placental Tissue

Only one rat from the 1.125 mg/kg dose level showed ^{14}C -activity in the placenta (0.8 ng/g). On the other hand, the placentas from all the gravid rats receiving 3.558 mg/kg/day exhibited ^{14}C -activity of an average of 1.2 ng/g.

E. Feces and Carcass

The feces and carcass contain nearly all the administered ^{14}C dose and it is likely that most of the activity is in the feces and the amount in the carcass was in the gastrointestinal tract. This is conjectural since the gastrointestinal tract was not removed and washed free of feces and the washings and tissue separately analyzed. The level of ^{14}C -activity was considerably less the third day after the administration of the last dose than detected on day-6 or day-15.

VII. Conclusions

- Low levels (maximum average value for the high dose: 0.97 ng/g) of ^{14}C -BTC were detected in the maternal blood following oral dosing with 1.125 mg/kg or 3.558 mg/kg on days-6 through 15 of gestation.
- Fetal absorption of ^{14}C -activity was low (maximum average for the low dose: 2.47 ng/g) and inconsistent. Individual values for pup from the same litter ranged from non-detectable to a maximum value of 13 ng/g.
- Virtually 100% of the administered dose was recovered in the maternal feces indicating that absorption of (^{14}C) BTC from the gastrointestinal tract was minimal. This observation was strengthened by the low levels observed in the maternal blood and urine samples.

Richard J. LaRosa
Norman Zinn

APPENDIX

TABLE I

Six Hour Blood Radioactivity as ng ¹⁴C-BTC/g BloodDOSE - 1.125 ng/kg/day

<u>Day-6</u>		<u>Day-15</u>		<u>Day-18</u>	
<u>Rat #</u>	<u>ng/g</u>	<u>Rat #</u>	<u>ng/g</u>	<u>Rat #</u>	<u>ng/g</u>
6	0	21	4.7	36	0.22
7	0	22	0.70	37	0
8	0	+23	0.75	38	0
9	0	24	0.54	*39	0.17
+10	<u>0</u>	25	<u>0.70</u>	40	<u>0</u>
Average					
<u>X</u>	0	<u>X</u>	1.5	<u>X</u>	0.08

DOSE - 3.558 ng/kg/day

<u>Day-6</u>		<u>Day-15</u>		<u>Day-18</u>	
<u>Rat #</u>	<u>ng/g</u>	<u>Rat #</u>	<u>ng/g</u>	<u>Rat #</u>	<u>ng/g</u>
11	0.8	26	0.83	*41	0
12	0	27	0.61	42	0.10
13	0	28	0.80	43	0.21
14	0	29	0.87	44	0.48
15	<u>0.3</u>	*30	<u>1.72</u>	45	<u>0.93</u>
Average					
<u>X</u>	0.2	<u>X</u>	0.97	<u>X</u>	0.34

+ Rat #10 - several hours after blood collection

* Non-gravid

TABLE II

Twenty-Four Hour Urine Radioactivity as ng ^{14}C -BTC/ml UrineDOSE - 1.125 mg/kg/day

<u>Day-6</u>			<u>Day-15</u>			<u>Day-18</u>		
<u>Rat #</u>	<u>ng/ml</u>	<u>ml Urine</u>	<u>Rat #</u>	<u>ng/ml</u>	<u>ml Urine</u>	<u>Rat #</u>	<u>ng/ml</u>	<u>ml Urine</u>
6	25	28	21	8	30	36	1	26
7	26	31	22	25	25	37	0	13
8	80	12	+23	77	5	38	1	33
9	28	32	24	23	18	39	3	17
+10	103	9	25	5	25	40	0	16
<u>X</u>	52	22	<u>X</u>	28	21	<u>X</u>	1	21

+ Rat died several hours after blood collection

DOSE - 3.558 mg/kg/day

<u>Day-6</u>			<u>Day-15</u>			<u>Day-18</u>		
<u>Rat #</u>	<u>ng/ml</u>	<u>ml Urine</u>	<u>Rat #</u>	<u>ng/ml</u>	<u>ml Urine</u>	<u>Rat #</u>	<u>ng/ml</u>	<u>ml Urine</u>
11	143	13	26	45	35	41	5	14
12	136	12	27	39	35	42	13	23
13	201	13	28	89	35	43	3	31
14	128	13	29	18	22	44	3	20
15	92	18	30	45	14	45	2	22
Avg. <u>X</u>	140	14	<u>X</u>	47	28	<u>X</u>	5	22

TABLE III

Six-Day Uterus Radioactivity as ng ^{14}C -BTC/Uterus

<u>DOSE: 1.125 mg/kg/day</u>		<u>3.558 mg/kg/day</u>	
<u>Rat No.</u>	<u>ng/Uterus</u>	<u>Rat No.</u>	<u>ng/Uterus</u>
6	0	11	1.59
7	1.53	12	0.87
8	2.53	13	0.68
9	0.39	14	0.73
10+	0.44	15	0.88
Avg.	0.98	Avg.	0.95

+ Rat died several hours after blood collection

TABLE IV

Day-15 and Day-18 Placental Radioactivity as ng ¹⁴C-BTC/g Placenta

DOSE: 1.125 mg/kg/day			3.558 mg/kg/day		
Day-15					
<u>Rat No.</u>	<u>ng/Placenta</u>	<u>ng/g Placenta</u>	<u>Rat No.</u>	<u>ng/Placenta</u>	<u>ng/g Placenta</u>
21	0	0	26	0	0
22	0	0	27	0	0
23	92	71	28	3.3	6.6
24	0	0	29	5.4	2.6
25	0	0	30*	---	---
			Avg.	4.35	4.6
Day-18					
<u>Rat No.</u>	<u>ng/Placenta</u>	<u>ng/g Placenta</u>	<u>Rat No.</u>	<u>ng/Placenta</u>	<u>ng/g Placenta</u>
36	0	0	41*	---	---
37	0	0	42	4.8	1.8
38	0	0	43	1.2	1.3
39	0*	0	44	1.9	1.1
40	2.1	0.8	45	2.1	0.7
			Avg.	2.5	1.23

* Not pregnant, uterus

Day-15 Fetal Radioactivity as ng ^{14}C -BTC/g Fetus

DOSE - 1.125 mg/kg/day

<u>Rat #21</u>			<u>Rat #23</u>			<u>Rat #24</u>			<u>Rat #25</u>		
<u>Fetus No.</u>	<u>ng/Fetus</u>	<u>ng/g Fetus</u>	<u>Fetus No.</u>	<u>ng/Fetus</u>	<u>ng/g Fetus</u>	<u>Fetus No.</u>	<u>ng/Fetus</u>	<u>ng/g Fetus</u>	<u>Fetus No.</u>	<u>ng/Fetus</u>	<u>ng/g Fetus</u>
1	0	0	1	0	0	1	0	0	1	0	0
2	0	0	2	0	0	2	0	0	2	0	0
3	0	0	3	0	0	3	0	0	3	0	0
4	0	0	4	0	0	4	0	0	4	0	0
5	0	0	5	0	0	5	0	0	5	0	0
6	0	0	6	0	0	6	0	0	6	0	0
7	0	0	7	0	0	7	0	0	7	0	0
8	0	0	8	0	0	8	0	0	8	0	0
9	0	0	9	0	0	9	0	0	9	0	0
10	0	0	10	0	0	10	0	0	10	0	0
11	0	0	11	0	0				11	0	0
12	0	0	12	0	0						
13	0	0									
14	0	0									
* \sum		0			0			0			0
\bar{X}		0			0			0			0

* Values based on those fetuses displaying ^{14}C -activity

Rat #22 - non-gravid

Day-15 Fetal Radioactivity as ng ^{14}C -BTC/g Fetus

DOSE - 3.550 mg/kg/day

Rat #26			Rat #27			Rat #28			Rat #29		
Fetus No.	ng/Fetus	ng/g Fetus	Fetus No.	ng/Fetus	ng/g Fetus	Fetus No.	ng/Fetus	ng/g Fetus	Fetus No.	ng/Fetus	ng/g Fetus
1	0	0	1	0	0	1	0	0	1	0	0
2	0	0	2	0	0	2	2.4	4.8	2	0.37	0.9
3	0	0	3	0	0	3	0.27	0.7	3	0.33	0.8
4	0	0	4	0	0				4	0.51	1.3
5	0.90	1.8	5	0	0				5	0.34	0.9
6	0	0	6	0	0				6	0.45	1.1
7	0.26	0.5	7	0	0				7	0.66	1.7
8	0	0	8	0	0				8	0.26	0.7
9	0.35	0.9	9	0	0				9	0.43	1.1
10	0	0	10	0	0				10	0.94	2.4
11	0	0	11	0	0				11	0.30	0.8
12	0	0							12	0.29	0.7
13	0	0							13	0.49	1.2
14	0	0							14	0.46	1.2
15	0.67	1.7							15	0	0
16	0	0							16	0.58	1.5
17	0	0									
* Σ		4.9	Σ		0	Σ		5.5	Σ		16.3
		1.23	\bar{X}		0	\bar{X}		2.75	\bar{X}		1.16

* Values based on these values distributed 14C

Day-18 Fetal Radioactivity as ng ¹⁴C-BTC/g Fetus

DOSE - 1.125 mg/kg/day

<u>Rat #36</u>			<u>Rat #37</u>			<u>Rat #38</u>			<u>Rat #40</u>		
<u>Fetus No.</u>	<u>ng/Fetus</u>	<u>ng/g Fetus</u>	<u>Fetus No.</u>	<u>ng/Fetus</u>	<u>ng/g Fetus</u>	<u>Fetus No.</u>	<u>ng/Fetus</u>	<u>ng/g Fetus</u>	<u>Fetus No.</u>	<u>ng/Fetus</u>	<u>ng/g Fetus</u>
1	0	0	1	0	0	1	0	0	1	0.22	0.06
2	0	0	2	0	0	2	3.1	13	2	0	0
3	0	0	3	0	0	3	0	0	3	0.19	0.036
4	0	0	4	0	0	4	0	0	4	0.19	0.052
5	0	0	5	0	0	5	0	0	5	23.00	0.064
6	0	0	6	0	0	6	0	0	6	0.45	0.13
7	0	0	7	0	0	7	0	0	7	0.20	0.056
8	0	0	8	0	0	8	0	0	8	0.10	0.03
9	0	0	9	0	0	9	0	0	9	0	0
10	0	0	10	8.1	6.8	10	0	0	10	0	0
11	5.9	4.5	11	0	0	11	0	0	11	0	0
12	0	0	12	0	0	12	0	0	12	0	0
13	0	0	13	0	0						
14	0	0	14	0	0						
* X		4.5 4.5			6.8 6.8			13.0 13.0			0.428 0.061

* Values based on those fetuses displaying ¹⁴C-activity

Rat #39 - non-gravid

TABLE VIII

Day-18 Fetal Radioactivity as ng $^{14}\text{C-DIC/g}$ Fetus

DOSE - 0.556 mg/kg/day

Rat #42			Rat #43			Rat #44			Rat #45		
Fetus No.	ng/Fetus	ng/g Fetus	Fetus No.	ng/Fetus	ng/g Fetus	Fetus No.	ng/Fetus	ng/g Fetus	Fetus No.	ng/Fetus	ng/g Fetus
1	0	0	1	0	0	1	0	0	1	0	0
2	0	0	2	0	0	2	0	0	2	0	0
3	0	0	3	0	0	3	0	0	3	0	0
4	0	0	4	0	0	4	0	0	4	0	0
5	0	0				5	0	0	5	0	0
6	0	0				6	0	0	6	0	0
7	0	0				7	0	0	7	0	0
8	0	0				8	0	0	8	0	0
9	0	0				9	0	0	9	0	0
10	0	0							10	0	0
11	0	0							11	0	0
12	0	0							12	0	0
13	0	0							13	0	0
14	0	0							14	0	0
15	0	0							15	0	0
* X		0			0			0			0
		0			0			0			0

* Values based on those fetuses displaying ^{14}C -activity

Rat #41 - non-gravid

TABLE IX

Twenty-Four Hour Feces Radioactivity as ug ^{14}C -BTC/g Feces

<u>DOSE 1.125 mg/kg/day</u>		<u>3.558 mg/kg/day</u>	
<u>Day-6</u>			
<u>Rat #</u>	<u>mcg ¹⁴C-BTC/g</u>	<u>Rat #</u>	<u>mcg ¹⁴C-BTC/g</u>
6	73.7	11	235.8
7	52.8	12	322.8
8	97.1	13	366.2
9	64.1	14	214.2
10	<u>48.3</u>	15	<u>237.9</u>
Avg.	$\frac{\Sigma}{X}$ 336.0 67.2	Avg.	$\frac{\Sigma}{X}$ 1376.9 275.38
<u>Day-15</u>			
21	33.9	26	123.0
22	107.4	27	203.4
23	13.7	28	213.6
24	62.4	29	199.2
25	<u>67.1</u>	30	<u>364.5</u>
Avg.	$\frac{\Sigma}{X}$ 284.5 56.9	Avg.	$\frac{\Sigma}{X}$ 1103.7 220.74
<u>Day-18</u>			
36	3.2	41	21.6
37	4.4	42	25.0
38	3.1	43	11.7
39	14.1	44	12.5
40	<u>3.6</u>	45	<u>12.4</u>
Avg.	$\frac{\Sigma}{X}$ 28.4 5.68	Avg.	$\frac{\Sigma}{X}$ 83.2 16.64

TABLE X

Carcass Radioactivity as ug ^{14}C -BTC/g Carcass

<u>DOSE</u> <u>1.125 mg/kg/day</u>		<u>3.558 mg/kg/day</u>	
<u>Day-6</u>			
<u>Rat #</u>	<u>mcg ¹⁴C-BTC/g</u>	<u>Rat #</u>	<u>mcg ¹⁴C-BTC/g</u>
6	0.297	11	0.611
7	0.360	12	0.799
8	0.079	13	0.591
9	0.347	14	1.797
10	<u>1.395</u>	15	<u>0.883</u>
Avg.	$\frac{\Sigma}{X}$ 2.47 0.496	Avg.	$\frac{\Sigma}{X}$ 4.681 0.936
<u>Day-15</u>			
21	0.015	26	0.285
22	0.333	27	1.484
23	1.440	28	0.278
24	0.098	29	0.391
25	<u>0.179</u>	30	<u>0.630</u>
Avg.	$\frac{\Sigma}{X}$ 2.06 0.413	Avg.	$\frac{\Sigma}{X}$ 3.068 0.614
<u>Day-18</u>			
36	0.002	41	0.065
37	0.020	42	0.049
38	0.015	43	0.022
39	0.005	44	0.019
40	<u>0.004</u>	45	<u>0.003</u>
Avg.	$\frac{\Sigma}{X}$ 0.171 0.034	Avg.	$\frac{\Sigma}{X}$ 0.158 0.032

M.C. 11
J. H. Hill
S. L. 1
Hyamine
1622

Distribution: Dr. W.E. Goode
Dr. W.R. Lyman
Dr. G.A. Miller
Mr. J.N. Moss
Mr. R.C. Richmond
Dr. M.C. Seidel
Mr. V.H. Unger
Dr. M.J. Williamson

January 7, 1974

cc: 4/24/74 J.M. Smith

Title: Dermal Absorption Study on ^{14}C Hyamine 1622

Project: 53-3477

Author: Matthew H. Watts

Objective: To determine whether labeled Hyamine 1622 is absorbed from the skin of rabbits following repeated applications.

Summary: A 10% (W/W) aqueous solution of ^{14}C Hyamine 1622 was applied to the skin of two rabbits, one with intact skin and the other with abraded skin. Application of the ^{14}C Hyamine 1622 was made on four consecutive days. Blood samples were taken from each animal at pre-selected time intervals on each day.

The blood samples were radioassayed for ^{14}C activity following combustion and liquid scintillation. The data were analyzed by Computer Data Reduction Program R 1020.

In general, trace quantities of ^{14}C activity were found in excess of background, however, a single sample assayed ^{14}C activity equivalent to 1.296 ppm ^{14}C Hyamine 1622. This level corresponds to 0.015% of the amount applied and may be an artifact because it was followed by three successive samples (28, 31 and 48 hour blood samples - abraded skin rabbit) in which no significant ^{14}C activity was detectable. The average concentrations were 0.1988 ppm in the blood from the rabbit with the abraded skin and 0.1658 ppm in the blood of the rabbit with the intact skin.

Conclusions: Trace quantities, based on the background count level, of topically applied ^{14}C Hyamine 1622 were observed in the blood under the conditions of this test protocol. However, the random nature of the occurrence of these trace quantities, as well as the lack of evidence of accumulation, strongly suggests that these points were artifactual.

Recommendations: The apparent occurrence of radioactivity in the blood indicating trace amounts of ^{14}C Hyamine 1622 prompts us to recommend a study of the effect of chronic application of Hyamine 1622 to a larger population of animals. Furthermore, a material balance study is also recommended.

Acknowledgement: Dr. A. Rothman, Develop. Ag. Chem. Res., for the ^{14}C Hyamine 1622.
Dr. M. Williamson, Analytical Research, for the radioassay work.

Work Done By: A. Benica, H. Mathason, M. Watts.

Date of Work: October 9-12, 1973.

Notebook Ref.: 31609-10.

Edited By: Jack N. Moss

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MEW:lmr

I. Introduction

Hyamine 1622 is a pure crystalline quaternary ammonium product which is readily formulated into solutions, powders, tablets, and proprietary pastes or ointments. In suitable formulations the Hyamine compounds contribute germicidal and deodorant properties to a variety of products for use in homes, restaurants, dairies and other food processing plants and for general industrial or veterinary disinfection. In pharmaceutical and cosmetic products, the Hyamine germicides contribute both deodorant and antiseptic properties.

Extensive studies have been made on the toxicology of Hyamine 1622 which shows a wide margin between germicidally effective concentrations and concentrations likely to be toxic or irritating. Little is known, however, about the absorption of Hyamine 1622 through the skin when it is formulated into cosmetics or pharmaceutical products. A dermal absorption study on ^{14}C Hyamine 1622 was undertaken to determine if the material was absorbed from the skin following topical application.

II. Method

A. Materials

^{14}C Hyamine 1622 Lot 79.01, Specific Activity = 58 microcuries/gram, 3.406 grams total sample supplied by Laboratory 23.

B. Animal Studies

The technique for applying the test sample to skin sites was similar to the classical method of Draize¹ for measuring primary skin irritants.

Two adult female albino rabbits weighing 2.5-3.0 kgs were used. Hair on the dorsal surface was removed by close clipping being careful to avoid nicking the skin. The skin of one rabbit was allowed to remain intact while the second one was abraded.

Two 1" x 1", double thickness gauze patches were applied to 2 test sites on each rabbit and secured with surgical tape. 0.5 ml of the radioactive test substance was introduced under each of the 2 patches. The sites of application were then wrapped with an occlusive rubber dam which was secured with a rigid leather harness. Twenty-four hours later, the occlusive dressing was removed and blood samples were taken from the marginal ear vein at pre-selected intervals. The application of test material and blood sampling was then repeated as described above for four consecutive days.

C. Blood Sampling

Sampling was made at 0, 2, 4 and 7 hours on the first day of application; 24, 48 and 31 hours on the 2nd day; 48, 52 and 55 hours on the third day; then at 72 and 77 hours after the first application of radioactive material.

The blood was withdrawn from a marginal ear vein which was dis-tended by manually stripping the hair immediately above the vein and then swabbing the area with xylene which was then wiped away. Successive bleedings were made from the same ear working from the distal to the proximal end. Approximately 1 cc of whole blood was removed and transferred to a preweighed combustion boat.

D. Analysis of Samples

Samples of blood were weighed into Zircon combustion boats and combusted in a furnace similar in design to that described by Peets, Flourini and Buyske². The furnace contained four Vycor tubes into which samples were placed. The combustion cycle required 90 minutes during which time the temperature rose in steps from room temperature to 1450°F . The combustion products were passed over a cupric oxide catalyst held at 1450°F to complete the conversion of any ^{14}C to $^{14}\text{CO}_2$ which was collected in a trap containing 10 ml of 5M ethanolamine in methyl cellosolve.

After the final trap volume was recorded, a 4 ml aliquot was transferred by pipette into a polyethylene counting vial. Fifteen ml of a methyl cellosolve/toluene base counting solution was added and the sample was counted on a Packard Instrument Co. Model 3315 liquid scintillation spectrometer. Instrument settings were:

Window	50-1000
Gain	30%

Fixed efficiency values were recently evaluated and entered into the computer program. The counting efficiency was fixed at 66.8 percent.

Control blood samples from untreated rabbits were combusted and counted along with the blood samples from treated rabbits to determine the control count rate. The combustion efficiency was fixed at 91.5 percent.

E. Calculations

The average count rate for a combustion-counting sample expressed as counts per minute (cpm) and corrected for the background count rate (cpm of control samples) was determined by the counting efficiency and converted to the corresponding disintegration rate (dpm). Based on a specific activity for ^{14}C Hyamine 1622 of 128 dpm per microgram the computer then determined the residue (ppm) of ^{14}C . In the calculation of all data the red channel was used. The average of 13 controls was 15.6 (Std. Dev. 1.4).

Blood Mass = $6.2\% \times \text{body weight (grams)}$
Abraded Rabbit Blood Mass = $2600 \text{ grams} \times 0.062 = 161.2 \text{ g.}$
Intact Rabbit Blood Mass = $2900 \text{ grams} \times 0.062 = 179.8 \text{ g.}$

References

1. Draize, J.H.; Woodard, G.; Calvery, H.O. - J. Pharm. & Exp. Therap. 82: 377 (1944).
2. Anal. Chem. 32:1465 (1960).

Table I
Radioassay of Blood From Intact Rabbit (2.9 kg) Treated With
 ^{14}C Hyamine 1622

<u>Time of Sampling</u>	<u>DPM Applied $\times 10^5$</u>	<u>Cumulative Total App. DPM $\times 10^5$</u>	<u>DPM per Gram of Blood</u>	<u>Calculated¹ Total DPM in Blood $\times 10^5$</u>	<u>% DPM of That Applied</u>	<u>% DPM of Cumulative Applied</u>	<u>Equivalent ppm ^{14}C Hyamine 1622</u>
0	1.28	1.28	NS ²	NS	-	-	-
2	-	1.28	NS	NS	-	-	-
4	-	1.28	NS	NS	-	-	-
7	-	1.28	8.8	0.0000158	0.0012	0.0012	0.0686
24	1.28	2.56 1.28	37.5	0.0000674	0.0053	0.0026	0.2917
28	-	2.56	18.9	0.0000340	0.0026	0.0013	0.1469
31	-	2.56	17.9	0.0000322	0.0025	0.0013	0.1397
48	1.28	3.84 2.56	13.1	0.0000236	0.0018	0.0006	0.1022
52	-	3.84	32.2	0.0000579	0.0045	0.0015	0.2506
55	-	3.84	40.3	0.0000725	0.0057	0.0019	0.3133
72	1.28	5.12 3.84	35.0	0.0000629	0.0049	0.0013	0.2724
77	-	5.12	52.0	0.0000935	0.0073	0.0018	0.4042

¹ Calculated as Mass of Rabbit Blood = 6.2% x body weight (grams)
H.H. Dukes, Physiology of Domestic Animals, 6th Edition, p. 61 (1947).

² N.S. = Not Significant - test for significance set at 2 counts above background.

Table II

Radioassay of Blood From Abraded Rabbit (2.6 kg) Treated With
 ^{14}C Hyamine 1622

Time of Sampling	DPM Applied $\times 10^6$	Cumulative Total App DPM $\times 10^6$	DPM per Gram of Blood	Calculated ¹ Total DPM in Blood $\times 10^6$	% DPM of That Applied	% DPM of Cumulative Applied	Equivalent ppm ^{14}C Hyamine 1622
0	1.28	1.28	16.0	0.0000258	0.0020	0.0020	0.1244
2	-	1.28	12.1	0.0000195	0.0015	0.0015	0.0941
4	-	1.28	51.7	0.0000833	0.0065	0.0065	0.4019
7	-	1.28	11.7	0.0000189	0.0015	0.0015	0.0909
24	1.28	2.56 1.28	116.8	0.0001883	0.0147	<u>0.0073</u>	1.296
28	-	2.56	NS ²	NS	-	-	-
31	-	2.56	NS	NS	-	-	-
48	1.28	3.84 2.56	NS	NS	-	-	-
52	-	3.84	15.3	0.0000247	0.0019	0.0006	0.1188
55	-	3.84	10.7	0.0000172	0.0013	0.0004	0.0834
72	1.28	5.12 3.84	8.2	0.0000132	0.0010	<u>0.0003</u>	0.0639
77	-	5.12	14.3	0.0000230	0.0018	0.0005	0.1117

¹Calculated as Mass of Rabbit Blood = 6.2% x body weight (grams)
 H.H. Dukes, Physiology of Domestic Animals, 6th Edition, p. 61 (1947).

²N.S. = Not Significant - test for significance set at 2 counts above background.

Table IIIRaw Data for Determination of Background from Control Blood Samples

Specific Activity for ^{14}C Hyamine 1622 128 DPM per microgram
 Counting Efficiency Fixed at 63.0 percent
 Combustion Efficiency Fixed at 93.0 percent
 Test for Non-Significance Set at 2 Counts Above Background.

Control Samples

Sample Ident.	Average Sample CPM			Sample Wgt. (Gms)
	Red	Green	Blue	
2910C0100	15.6	18.0	11624.5	1.093
2910C0200	15.2	18.5	11460.5	1.583
2910C0300	14.5	16.7	11696.7	1.061
2910C0400	15.2	17.7	11888.2	0.966
2910C0500	16.6	19.3	11250.9	1.780
2910C1200	15.2	18.0	11359.6	1.220
2910C1300	16.2	18.2	11845.1	1.068
2910C1524	19.7	21.8	11717.5	1.120
2910C1624	14.2	17.0	11440.5	0.989
2910C1848	14.2	18.2	11566.4	1.353
2910C1948	15.9	18.3	11683.6	1.110
2910C2172	15.1	17.7	11376.4	1.464
2910C2272	15.6	18.4	11593.9	1.132
Average	15.6	18.3	11577.2	
Std. Dev.	1.4	1.2	192.3	
No. Samples	(13)	(13)	(13)	

¹ 2910 Notebook Reference; C = control; 01 = Sample Number;
 00 Sampling Hour.

Table IV

Raw Data for Determination of DPM & ppm of Abraded and Intact
Animal Blood Samples

Treated Samples

Sample Ident. ¹	Avg. Samp. CPM	Sample* DPM	Samp. Wgt. (Gms)	DPM** per Gm	Residue (ppm)
Subgroup					
2910TIA00	14.7	NS	0.942	NS	NDR
2910TAA00	19.2	5.6	0.953	16.0	0.1244
2910TIA02	17.2	NS	1.072	NS	NDR
2910TAA02	18.8	4.9	1.107	12.1	0.0941
2910TIA04	16.9	NS	1.137	NS	NDR
2910TAA04	31.4	25.0	1.301	51.7	0.4019
2910TIA07	18.1	3.8	1.180	8.8	0.0686
2910TAA07	18.6	4.6	1.074	11.7	0.0909
2910TIA24	26.3	16.9	1.213	37.5	0.2917
2910TAA24	51.1	56.3	0.908	166.8	1.296
2910TIA28	20.7	8.1	1.153	18.9	0.1469
2910TAA28	17.2	NS	1.001	NS	NDR
2910TIA31	20.5	7.6	1.149	17.9	0.1397
2910TAA31	17.3	NS	0.922	NS	NDR
2910TIA48	18.6	4.7	0.966	13.1	0.1022
2910TAA48	17.4	NS	1.111	NS	NDR
2910TIA52	24.4	13.8	1.152	32.2	0.2506
2910TAA52	20.0	6.9	1.221	15.3	0.1188
2910TIA55	26.5	17.3	1.154	40.3	0.3133
2910TAA55	18.4	4.3	1.090	10.7	0.0834
2910TIA72	28.6	20.6	1.579	35.0	0.2724
2910TAA72	17.8	3.4	1.130	8.2	0.0639
2910TIA76	30.6	23.7	1.228	52.0	0.4042
2910TAA76	19.6	6.2	1.160	14.3	0.1117
Average	22.1			23.4	0.1823
Std. Dev.	7.7			34.5	0.2681
No. Samples	(24)			(24)	(24)

*Corrected for background and avg. counting efficiency (63.0 pct.)

**Corrected for counting aliquot (4 ml of 10 ml), and combustion efficiency (93.0 pct.)

NS = Not Significant

NDR = No Detectable Residue

¹2910 Notebook Reference; T = Treated; IA = Intact; AA = Abraded;
00 Sampling Hour.

NATIONAL Toxicology Program (NTP) (1984). The Absorption, Distribution and elimination of ^{14}C -Benzethonium chloride Following IV Administration or a single or a 10-day ^{Repeated} Dermal Application in Fischer 344 Rats.

ABSTRACT

Benzethonium chloride is used widely in cosmetic formulations and pharmaceutical preparations. The objectives of this study were to determine the extent and rate of percutaneous absorption, pattern of tissue distribution, and route of elimination of benzethonium chloride in male Fischer 344 rats. Treatment groups consisted of a single intravenous (IV) dose group 0.15 mg/Kg (9.77 $\mu\text{Ci/mL}$); single dermal application low, 0.15 mg/Kg, (9.77 $\mu\text{Ci/mL}$) or high, 1.5 mg/Kg (48.9 $\mu\text{Ci/mL}$) dose group; and a 10-day repeated single daily dermal application high dose group, 1.5 mg/Kg that was treated on Day 11 with a 1.5 mg/Kg dose of ^{14}C -benzethonium chloride (48.9 $\mu\text{Ci/mL}$). Following IV administration, the whole blood ^{14}C -benzethonium chloride equivalents concentration time curve was biexponential with a short distribution phase. Pharmacokinetic parameters determined for benzethonium chloride included a terminal volume of distribution $V_d(\beta)$ of 2.3 L/Kg, a clearance of 14.8 mL/min/Kg, and a half-life of 110.2 ± 8.0 min. After 24 hours, 3.6 ± 0.7 and 46.4 ± 10.8 percent of the administered dose was eliminated in the urine and feces. Following dermal application of ^{14}C -benzethonium chloride, a non-occlusive dressing was placed over the application site. After a 24-hour contact period, the application site was washed with distilled water to remove nonabsorbed test chemical. Whole blood samples collected after a single or repeated application of ^{14}C -benzethonium chloride equivalents were generally below detection limits (3.26×10^{-3} $\mu\text{g/mL}$). Peak elimination of ^{14}C -benzethonium chloride equivalents in the urine and feces was observed between 24 to 48 and 48 to 72 hours, respectively. Total urinary excretion accounted for 1 to 2 percent of the actual applied dose. Total fecal excretion accounted for approximately 45 percent of the actual applied dose following a single dermal application and approximately 25 percent of the last dose following 10 days of repeated dermal application. Tissue levels of ^{14}C -benzethonium chloride equivalents were generally below detection limits (3.60 $\mu\text{g/g}$). Low levels were measured in the liver, while levels in the application site skin and residual carcass ranged from approximately 10 to

50 percent and 1 to 15 percent, respectively, throughout the 7-day collection period. Skin layer analysis of the application site 24 hours after dosing showed that over 99 percent of the total ^{14}C -benzethonium chloride equivalents were in the epidermis. Based upon the percentage of ^{14}C -benzethonium chloride equivalents recovered in the urine, feces and tissues (including the application site skin) the total bioavailability was approximately 60 and 56 percent for the single low and high dose dermal application groups, respectively, whereas a value of approximately 36 percent was determined for the repeated dermal application group.

This report has been prepared by a laboratory that conducted these studies under the direction and support of the National Toxicology Program. The contents and conclusions have not been reviewed by the NTP staff at this time and therefore do not necessarily represent the position of the NTP.

DISCUSSION

This study characterized the extent of absorption, pattern of tissue distribution, and pathway of elimination following a single intravenous administration or single or 10-day repeated dermal application of benzethonium chloride in male Fischer 344 rats. Background literature pertaining to pharmacokinetic disposition data about benzethonium chloride is limited to a fetal absorption study. La Rosa et al., (1978) gave pregnant rats an oral dose of ^{14}C -benzethonium chloride on days 6 through 15 of gestation and reported variable fetal absorption, with the majority of the ^{14}C -benzethonium chloride equivalents remaining within the dam. Thus, the present study was performed due to the limited amount of pharmacokinetic information about benzethonium chloride and necessity for this type of information for performing chemical safety evaluation studies.

Following a single IV injection, a plot of the concentration in the blood over time produced a biexponential curve. Pharmacokinetic modeling of this data showed benzethonium chloride was best fitted to a two compartment model which indicated it is readily distributed into secondary compartments (tissues) from the central compartment (systemic circulation). The calculated volume of distribution for benzethonium chloride (2.3 L/Kg) indicates that distribution exceeds the volume of the systemic circulation. Thus, benzethonium chloride infiltrates interstitial cellular spaces and peripheral tissues. The clearance of benzethonium chloride from blood is a function of the hepatic blood flow and efficiency of benzethonium chloride to be extracted by the liver, as evidenced by primarily fecal excretion. The experimentally determined clearance value, which was less than hepatic blood flow (66 mL/min/Kg, King et al., 1986) suggests that benzethonium chloride is cleared relatively inefficiently from the blood. However, it is unlikely this would have any serious toxicokinetic implications under normal conditions since the half life of benzethonium chloride was approximately 2 hours. Thus, benzethonium chloride is rapidly removed from the central compartment with over 90 percent eliminated within approximately 8 hours.

Based upon the amount recovered in the excreta and tissues, benzethonium chloride was readily absorbed through the skin. However, the

rate of penetration was slow and erratic as shown by the low circulating blood levels in animals following dermal application of benzethonium chloride. The major barrier of chemicals to absorption by the percutaneous route of administration is the stratum corneum of the epidermis. Diffusion of benzethonium chloride through the densely packed layer of keratinized cells that comprise the stratum corneum is generally the rate-limiting step in percutaneous absorption. Once through the epidermal layers, the dermis, which is primarily composed of an aqueous matrix, does little to impede diffusion of the chemical into the systemic circulation. In this study, analysis of ^{14}C -benzethonium chloride equivalents in discrete layers of the application site skin support the physiochemical and biological properties that govern dermal absorption. Virtually all of the benzethonium chloride in the application site skin was found in the upper portion of the first skin slice (0 to 500 microns) which would include all layers of the epidermis (Bronaugh and Maibach, 1983). Only slight amounts of benzethonium chloride were measured deep in the skin. This skin slice would include the stratum corneum and penetration of the test chemical through the skin may have been impeded at this layer. Only low levels of ^{14}C -benzethonium chloride equivalents were measured in the deep layers of the skin. Thus, benzethonium chloride appears to diffuse into the epidermis where it may become concentrated at the level of the stratum corneum. The epidermis then acts as a depot for slow continual passage of benzethonium chloride from the skin. Benzethonium chloride is leached from the depot and penetrates through the remaining dermal layers until it eventually reaches the systemic circulation.

The permeability of the skin to a test chemical may vary under a number of conditions (e.g., skin site, integrity, degree of hydration) and be affected by a variety of factors (e.g., dose, application site, species vehicle) (Klaassen, 1986). In this study, ethanol was chosen as the vehicle because it is extensively used in formulating benzethonium chloride topical preparations. The extent of absorption of benzethonium chloride in ethanol may underestimate the bioavailability of benzethonium chloride prepared in water-like formulations. The former dries the skin which decreases permeability, while the latter promotes hydration and enhances absorption. Another important factor to consider when using the extent of absorption

determined from this study for toxicological purposes, is the species. In general, rat skin is far more permeable to most chemicals than is human skin (Bronaugh and Maibach, 1983).

The major pathway of elimination of benzethonium chloride, following intravenous administration or dermal application, was the feces. These findings are consistent with the anticipated route of elimination in the rat based upon the molecular weight (mw) of benzethonium chloride (448). Small molecules ($mw < 350$) are excreted primarily in the urine, whereas larger molecules ($mw > 450$) are eliminated in the bile (Hirom, et al., 1972). Although benzethonium chloride is probably readily filtered by the glomerulus, based upon molecular size and chemical structure (quaternary nitrogen), it is likely that the majority of the benzethonium chloride is reabsorbed in the tubules and only a small percentage is ultimately excreted in the urine. The majority of the benzethonium chloride is removed from the systemic circulation by the liver. Biotransformation processes that take place in the liver possibly involve conjugation to a more polar metabolite which is then excreted in the bile.

The importance of the urinary route of elimination of benzethonium chloride from the body is difficult to evaluate based upon the results from this study. Interaction of benzethonium chloride with the glass metabolism cage precluded measuring ^{14}C -benzethonium chloride equivalent levels in the urine. Although the urinary system may not play a major role in the elimination of benzethonium chloride based upon the molecular weight of the test chemical, it does appear to be a minor pathway for excretion of benzethonium chloride. Furthermore, it is interesting to note that chemicals with molecular weights between 350 and 450 may be eliminated in the urine or feces if one or the other pathway is compromised (Hirom, et al., 1976).

Deposition of benzethonium chloride in the skin and penetration into the systemic circulation may have important toxicological implications. A review of the biochemical, cellular, and tissue effects of benzethonium chloride have been recently reviewed by CIR (1985). The mechanism credited to benzethonium chloride's antimicrobial activity (e.g., cell membrane disruption, protein denaturation, enzyme inhibition, alteration of activating enzymes, interference with cellular growth) may also produce localized or

systemic cellular injury. Previous acute (Finnegan and Dienna, 1953; Draize and Kelley, 1952; Arro and Salenstedt, 1973; Paniaqua, et al., 1961) and sub-chronic (FDA, 1979; Swan, 1944; Hamburger, 1968) toxicity study findings have been limited to localized irritation and fur depigmentation. Recent 14-day repeated dose and subchronic dermal toxicity studies support and extend the findings of these earlier studies (Hejtmancik et al., 1985a; 1985b). In the 14-day repeated dose study, male and female F344 rats received daily (5 times/week) dermal applications of benzethonium chloride in ethanol (vehicle control) in doses ranging from 4.0 to 100 mg/Kg of benzethonium chloride. Doses above 25 mg/Kg produced signs of systemic toxicity (e.g., decreased body weights, food consumption, and organ weights). Microscopic examination of the application site revealed chronic inflammation at the high doses and hyperkeratosis in the lower doses, e.g., <12 mg/Kg. In the sub-chronic toxicity study, benzethonium chloride was applied 5 times a week for 13 weeks in doses ranging from 1.5 to 25 mg/Kg. Local toxic effects of benzethonium chloride observed in all dose groups included chronic dermatitis, hyperkeratosis, and acanthosis. The benzethonium chloride-induced thickening of the skin may explain the difference in bioavailability between the single and repeated dermal application groups in this study. Thus, repeated dermal exposure may decrease the permeability of the skin and, thereby, the extent of absorption.

The results of this study suggest that benzethonium chloride, following single or repeated daily dermal applications, is absorbed through the skin and into the general circulation. Absorption into the skin was virtually complete within 6 hours after application. The epidermis of the skin impedes the rate of penetration but also serves as a depot for benzethonium chloride, from where it slowly penetrates into the general circulation. Once in the blood, benzethonium chloride is readily distributed throughout the major organs of the body. The major pathway of elimination is in the feces, but the urine may serve as a minor pathway for elimination. Based upon recovery of ^{14}C -benzethonium chloride equivalents in the urine, feces, and tissues (including the application site skin), the bioavailability of benzethonium chloride following a 24-hour contact period was approximately 55 to 60 percent following a single application and 35 percent following single daily repeated dermal applications.

REFERENCES

- Arro, L. and Salenstedt, C. R. (1973) Evaluation of toxicity of some quaternary ammonium compounds. *J. Biol. Stand.* 1(1):87.
- Bronaugh, R. L. and Maibach, H. I. (1983). *In vitro* percutaneous absorption. In: *Dermatotoxicology*. (Eds. Marzulli, F. N. and Maibach, H. I.), 2nd edition. McGraw-Hill International Book Co., p 117.
- CIR. (1985) Tentative Report of the Safety Assessment of Benzethonium Chloride and Methylbenzethonium Chloride. *Cosmetic Ingredients Review*, Washington, DC, 5:13.
- Draize, J. H. and Kelley, E. A. (1952) Toxicity to eye mucosa of certain cosmetic preparations containing surface-active agents. *The Toilet Goods Assoc., Proc. Sci. Sect.* 17:1.
- Dreisbach, R. H. (1980) *Handbook of Poisoning: Prevention, Diagnosis, and Treatment*. Los Altos, CA. Lange Medical Publications.
- FDA. (1979) Information copy of OTC topical antifungal report. Division of OTC Drug Evaluation (HFD-510), Bureau of Drugs.
- Finnegan, J. K. and Dienna, J. B. (1953) Toxicological observations on certain surface active agents. *The Toilet Goods Assoc., Proc. Sci. Sect.* 20:16.
- Homburger, F. (1968) Carcinogenicity of several compounds. *Nat. Tech. Inf. Ser. PB. No. 183 027*, p. 26.
- Hejtmancik, M., Placke, M., Ryan, M., Peters, A., and Athey, P. (1985). Thirteen-week subchronic dermal toxicity study of benzethonium chloride (CAS No. 121-54-0) in Fischer 344 rats. A Final Report to the National Toxicology Program. Battelle Columbus Laboratories, Columbus, Ohio.
- Hejtmancik, M., Ryan, M. J., Peters, A. C., and Athey, P. (1985). Dermal repeated dose study of benzethonium chloride (CAS No. 121-54-0) in Fischer 344 Rats: A Final Report to the National Toxicology Program. Battelle Columbus Laboratories, Columbus, Ohio.
- Hirom, P. C., Millburn, P., and Smith, R. L. (1976) Bile and urine as complementary pathways for the excretion of foreign organic compounds. *Xenobiotica* 6:55.
- Hirom, P. C., Millburn, P. Smith, R. L., and Williams, R. T. (1972) Species variations in the threshold molecular weight factor for the biliary excretion of organic anions. *Biochem. J.* 129:1071.
- King, F. G., Dedrick, R. L., and Farris, F. F. (1986) Physiological pharmacokinetic modeling of cis-dichlorodiammine platinum (II) (DDP) in several species. *J. Pknetics and Biopharm.* 14(1):131.

Klaassen, C. D. (1986) Distribution, excretion, and absorption of toxicants. In: Toxicology. The Basic Science of Poisons. (Eds. C. D. Klaassen, M. O. Amdur, and J. Doull). MacMillan Publishing Co., New York, p. 33.

Kligman, A. M. and Leyden, J. J. (1979) Reactions to standard patch test materials. Acta Derm.-Venereol. (Sweden) 59 (Supp 85):101.

LaRosa, R. T., Fine, N., and DeSalva, S. S. (1978) Rat maternal and fetal absorption of ^{14}C -benzethonium chloride (^{14}C -benzethonium chloride). Pharmacologist 20:254.

Liebert, M. A. (1985) Final Report on the safety assessment of benzethonium chloride and methyl-benzethonium chloride. J. Am. College Toxicol. 4(5):65.

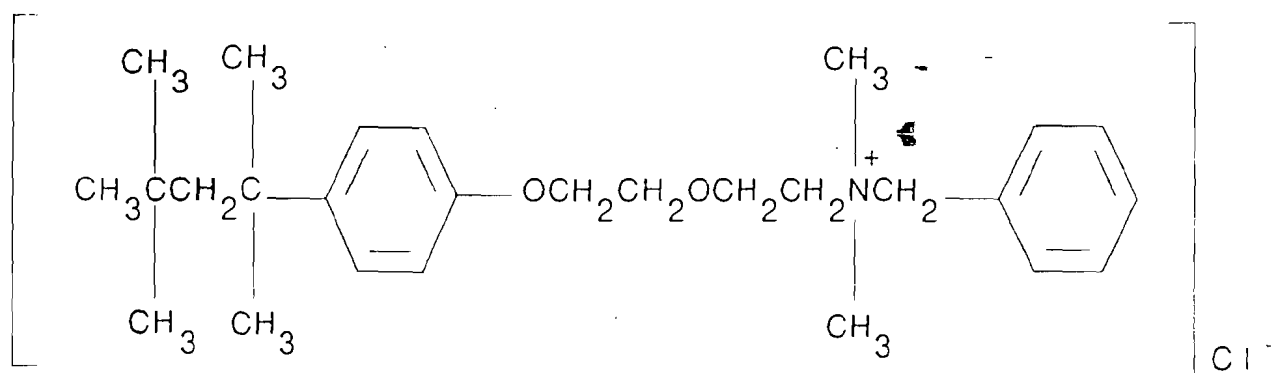
Little, A. D. (1986) Health and Safety Package for Benzethonium Chloride. National Toxicology Program.

Osol, A. (1980) Penningtons Pharmaceutical Sciences. Sixteenth edition. Easton, PA. Mack Publishing Co.

Paniaqua, M. E., Rio Piedrias, P. R., Valliant, A. B., and Gamble, C. J. (1961) Field trial of a contraceptive foam in Puerto Rico. J. Am. Med. Assoc., 177:125.

Swan, K. C. (1944) Reactivity of the ocular tissues to wetting agents. Am. J. Ophthalmol. 27:1118.

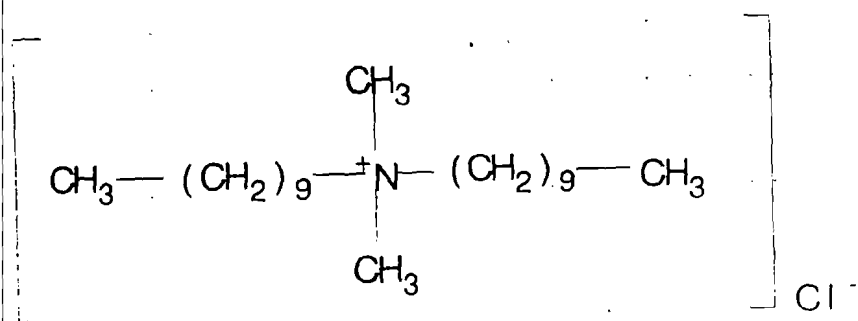
Structure of Benzethonium Chloride



Structure of DDAC and ADBAC

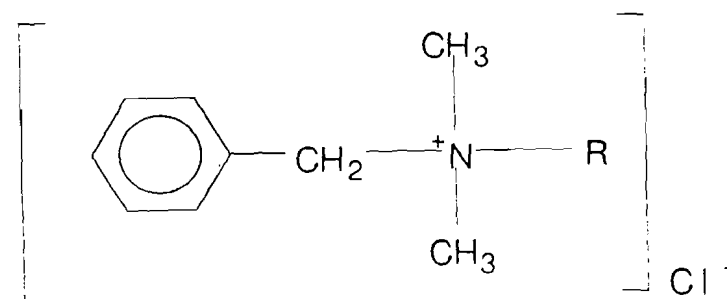
DDAC

Didecyldimethylammoniumchloride



ADBAC

Alkyldimethylbenzylammoniumchloride



R = C₁₂H₂₅ (40%)
C₁₄H₂₉ (50%)
C₁₆H₃₃ (10%)

Comparative Toxicity Data

Study Results for:

Study	ADBAC	DDAC	Benzethonium Chloride
Primary Eye Irritation			
Maximum Score (max 110)	19.5	37.5	13.5
Days to Recovery (max 21)	10	no recovery	14
Toxicity Category	II	I	II
Rat Acute Oral Toxicity (LD ₅₀)	430 mg/kg	238 mg/kg	420 mg/kg
Skin Sensitization	negative	negative	negative
Rat Teratology (NOEL)	10 mg/kg	1 mg/kg	100 mg/kg
Mutagenicity			
Ames Test	negative	negative	negative
Chromosomal Aberration	negative	negative	negative
ADME (oral administration)	89 - 99%	87 - 99%	Approx. 100%
	Radioactivity Recovered in Feces	Radioactivity Recovered in Feces	Radioactivity Recovered in Feces

TRS
Toxicology/Regulatory Services

SUMMARY OF COMPLETED STUDIES ON ADBAC QUAT¹

May 2, 1995

MAMMALIAN TOXICOLOGY STUDIES

1. Repeated Eye Instillation Study in Rabbits of Alkyl Dimethyl Benzyl Ammonium Chloride (ADBAC)

Performing Laboratory: Hill Top Biolabs, Inc.
Final Report Issued: 11/08/88
Treatment Concentrations: 2.5, 5.0 ppm
Study Duration: 3 weeks
Purpose: To evaluate the eye irritation potential of ADBAC at concentrations simulating swimming pool use
Results: No eye irritation was observed.

2. Photoallergy Study in Guinea Pigs

Performing Laboratory: Hill Top Biolabs, Inc.
Final Report Issued: 11/28/88
Study Design: Buehler method at 0.25%
Purpose: EPA registration (Guideline Reference No. 81-6)
Results: There was no evidence of photoallergy or contact sensitization.

¹ Study added since last summary (12/06/94) is marked with an asterisk (*).

Summary of Completed Studies on ADBAC Quat

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3. Two-Week Skin Irritation Study with Alkyl Dimethyl Benzyl Ammonium Chloride (ADBAC) in Rats

Performing Laboratory: Bushy Run Research Center
Final Report Issued: 10/05/88
Treatment Concentrations: 0.03, 0.1, 0.3, 1.0, 3.0, 6.0, 10.0%
Purpose: To determine the gradation of skin effects as a function of concentration following repeated dermal application
Results: No irritation was observed at concentrations $\leq 0.1\%$; minimal to moderate irritation was observed at 3.0%. At concentrations $\geq 6.0\%$ marked irritation was observed. No systemic toxicity was observed at any level.

4. Ninety-Day Subchronic Dermal Toxicity Study with Alkyl Dimethyl Benzyl Ammonium Chloride (ADBAC) in Rats

Performing Laboratory: Bushy Run Research Center
Final Report Issued: 05/14/90
Treatment Concentrations/
Dosage Levels: 0, 0.1, 0.3, 1.0% (w/w) corresponding to dosage levels of 0, 2, 6, 20 mg/kg b.w./day, respectively
Purpose: EPA registration (Guideline Reference No. 82-3)
Results: Slight skin irritation was noted in all treatment groups. No systemic toxic effects were observed at any dose level. Highest concentration represents the maximum dose of ADBAC which could be evaluated in this test system.

5. Evaluation of ADBAC in a Two-Week Palatability Study in Dogs

Performing Laboratory: International Research and Development Corporation
Final Report Issued: 08/03/93
Dietary Concentrations: 0, 400, 1200, 2400, 4000 ppm ($\approx 10, 30, 60$ and 100 mg/kg/day, respectively)
Purpose: To determine the maximum dietary concentration of ADBAC which will be tolerated by beagle dogs
Results: All animals survived to study termination. Diet rejection, diarrhea and weight loss were observed for animals in the 2400 and 4000 ppm dose groups. ADBAC dietary concentrations ≥ 2400 ppm were found to exceed the level of palatability in the beagle dog.

Summary of Completed Studies on ADBAC Quat

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6. Evaluation of ADBAC in a Two-Week Gavage Study in Dogs

Performing Laboratory: International Research and Development Corporation
Final Report Issued: 09/03/93
Dosage Levels: 0, 10, 30, 60, 100 mg/kg/day
Purpose: To determine the maximum dosage of ADBAC that could be tolerated by dogs when administered by gavage as an aqueous slurry mixed with the basal diet
Results: Clear signs of toxicity related to local irritation of the gastrointestinal tract were observed for animals in the 60 and 100 mg/kg/day groups. Similar but less severe changes were observed for animals in the 10 and 30 mg/kg/day groups. Based on the results of the study, dosage levels ≥ 60 mg/kg/day of ADBAC administered by gavage as an aqueous slurry mixed with basal diet were considered to exceed the dosage that could be tolerated in a chronic study.

7. Evaluation of ADBAC in an Eight-Week Dietary Toxicity Study in Dogs

Performing Laboratory: International Research and Development Corporation
Final Report Issued: 07/21/94
Dietary Concentrations: 0, 400, 800, 1200, 1600 ppm
Purpose: To obtain data from which to select dietary concentrations for a chronic toxicity study in dogs
Results: No clinical signs of toxicity were observed at any of the dietary concentrations. However, slightly reduced body weight gains and cholesterol values were observed in animals at the 1200 and 1600 ppm dietary concentrations. There were no necropsy or microscopic findings or changes in hematology which were attributed to treatment with ADBAC.

8. Evaluation of ADBAC in a One-Year Chronic Dietary Toxicity Study in Dogs

Performing Laboratory: International Research and Development Corporation
Final Report Issued: 05/03/94
Dietary Concentrations: 0, 120, 400, 1200 ppm
Purpose: EPA registration (Guideline Reference No. 83-1(b))
Results: Clear effect (1200 ppm) and no-effect (400 ppm) levels were determined for systemic toxicity. No specific target organ toxicity was observed and treatment had no effect on survival.

Summary of Completed Studies on ADBAC Quat

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9. Ninety-Day Dietary Dose Range Finding Study with Alkyl Dimethyl Benzyl Ammonium Chloride (ADBAC) in Mice

Performing Laboratory: Bushy Run Research Center
Final Report Issued: 04/15/88
Dietary Concentrations: 0, 100, 500, 1000, 4000, 8000 ppm
Purpose: To obtain data from which to select dose levels for long term studies
Results: Clear effect (≥ 4000 ppm) and no-effect (500 ppm) levels were determined. An equivocal effect on body weight was observed at the 1000 ppm dosage level. Dose response was found to be very steep. Further elucidation of dose response was needed between the 1000 and 4000 ppm levels.

10. Two-Week Dietary Dose Range Finding Study with Alkyl Dimethyl Benzyl Ammonium Chloride (ADBAC) in Mice

Performing Laboratory: Bushy Run Research Center
Final Report Issued: 09/01/88
Dietary Concentrations: 0, 2000, 3000 ppm
Purpose: To elucidate dose response between the 1000 and 4000 ppm levels
Results: Gradation of effects between 1000 and 4000 ppm was defined.

11. Chronic Dietary Oncogenicity Study with Alkyl Dimethyl Benzyl Ammonium Chloride (ADBAC) in Mice

Performing Laboratory: Bushy Run Research Center
Final Report Issued: 01/09/91
Dietary Concentrations: 0, 100, 500, 1500 ppm
Purpose: EPA registration (Guideline Reference No. 83-2)
Results: Clear effect (1500 ppm) and no-effect (≤ 500 ppm) levels were determined. No specific target organ toxicity was observed and treatment did not have any effect on survival or tumor incidence.

Summary of Completed Studies on ADBAC Quat

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12. Developmental Toxicity Dose Range-Finding Study of Alkyl Dimethyl Benzyl Ammonium Chloride (ADBAC) Administered by Gavage to CD® Rats

Performing Laboratory: Bushy Run Research Center
Final Report Issued: 01/20/93
Dosage Levels: 0, 25, 50, 100, 200, 400 mg/kg/day
Purpose: To obtain data from which to select dosage levels for a definitive developmental toxicity study
Results: Maternal toxicity was observed at dose levels ≥ 100 mg/kg/day. No effect on fetal toxicity was observed. In addition, treatment had no effect on gestational parameters or on the incidence of external variations or malformations. Based on these findings, dosage levels of 10, 30 and 100 mg/kg/day were selected for the definitive teratology study.

13. Developmental Toxicity Evaluation of Alkyl Dimethyl Benzyl Ammonium Chloride (ADBAC) Administered by Gavage to CD® Rats

Performing Laboratory: Bushy Run Research Center
Final Report Issued: 06/08/92
Dosage Levels: 0, 10, 30, 100 mg/kg/day
Purpose: EPA registration (Guideline Reference No. 83-3)
Results: Clear effect (100 mg/kg/day) and no-effect (10 mg/kg/day) levels were determined for maternal toxicity. Treatment had no effect on gestational parameters or incidences of variations or malformations.

Summary of Completed Studies on ADBAC Quat

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14. Developmental Toxicity Dose Range-Finding Study of Alkyl Dimethyl Benzyl Ammonium Chloride (ADBAC) Administered by Gavage to New Zealand White Rabbits

Performing Laboratory: Bushy Run Research Center
Final Report Issued: 03/09/93
Dosage Levels: 0, 1, 3, 10, 30, 60 mg/kg/day
Purpose: To obtain data from which to select dosage levels for a definitive developmental toxicity study
Results: Maternal toxicity was observed at dosage levels of 10, 30 and 60 mg/kg/day. No effect on fetal toxicity was observed. In addition, treatment had no effect on gestational parameters or on the incidence of external variations or malformations. Based on these findings, dosage levels of 1, 3 and 9 mg/kg/day were selected for the definitive teratology study.

15. Developmental Toxicity Evaluation of Alkyl Dimethyl Benzyl Ammonium Chloride (ADBAC) Administered by Gavage to New Zealand White Rabbits

Performing Laboratory: Bushy Run Research Center
Final Report Issued: 07/08/92
Dosage Levels: 0, 1, 3, 9 mg/kg/day
Purpose: EPA registration (Guideline Reference No. 83-3)
Results: Clear effect (9 mg/kg/day) and no-effect (3 mg/kg/day) levels were determined for maternal toxicity. Treatment had no effect on gestational parameters or incidences of variations or malformations.

16. Ninety-Day Dietary Toxicity Study with Alkyl Dimethyl Benzyl Ammonium Chloride (ADBAC) in Rats

Performing Laboratory: Bushy Run Research Center
Final Report Issued: 06/20/88
Dietary Concentrations: 0, 100, 500, 1000, 4000, 8000 ppm
Purpose: To obtain data from which to select dosage levels for long term studies
Results: Clear effect (4000 ppm) and no-effect (500 ppm) levels were determined. An equivocal effect on body weight was observed at the 1000 ppm dosage level. Dose response was found to be very steep. Further elucidation of dose response was needed between the 1000 and 4000 ppm levels.

Summary of Completed Studies on ADBAC Quat

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17. Two-Week Dietary Dose Range Finding Study with Alkyl Dimethyl Benzyl Ammonium Chloride (ADBAC) in Rats

Performing Laboratory: Bushy Run Research Center
Final Report Issued: 08/31/88
Dietary Concentrations: 0, 2000, 3000 ppm
Purpose: To elucidate dose response between the 1000 and 4000 ppm levels
Results: Gradation of effects between 1000 and 4000 ppm was defined.

18. Two-Generation Reproduction Study in Sprague-Dawley (CD®) Rats with Alkyl Dimethyl Benzyl Ammonium Chloride (ADBAC) Administered in the Diet

Performing Laboratory: Bushy Run Research Center
Final Report Issued: 01/30/90
Dietary Concentrations: 0, 300, 1000, 2000 ppm
Purpose: EPA registration (Guideline Reference No. 83-4)
Results: Clear effect (2000 ppm) and no-effect (≤ 1000 ppm) levels were determined for parental and neonatal toxicity. Treatment did not have an effect on any of the reproductive parameters.

19. Chronic Dietary Toxicity/Oncogenicity Study with Alkyl Dimethyl Benzyl Ammonium Chloride (ADBAC) in Rats

Performing Laboratory: Bushy Run Research Center
Final Report Issued: 07/08/91
Dietary Concentrations: 0, 300, 1000, 2000 ppm
Purpose: EPA registration (Guideline Reference No. 83-5)
Results: Clear effect (2000 ppm) and no-effect (1000 ppm) levels were determined. No specific target organ toxicity was observed and treatment did not have any effect on survival or tumor incidence.

20. Absorption, Distribution, Metabolism and Excretion Studies of Alkyl Dimethyl Benzyl Ammonium Chloride (ADBAC) in the Rat

Performing Laboratory: Biological Test Center
Final Report Issued: 01/26/89
Study Design: The following 4 treatment regimens were evaluated:
1. single low-dose oral (10 mg/kg)
2. repeated low-dose oral (10 mg/kg)
3. single high-dose oral (50 mg/kg)
4. low-dose i.v. (10 mg/kg)
Purpose: EPA registration (Guideline Reference No. 85-1)
Results: Following oral administration, 87 - 99% of the administered ¹⁴C-ADBAC was excreted in the feces and 5 - 8% was excreted in the urine. No bioaccumulation was noted. Metabolism in the gut proceeded via oxidation of alkyl side chain. All metabolites were considered to be less toxic than the parent compound. ADBAC was found to be widely distributed in tissues following i.v. administration with approximately 45 - 55% in the feces, 20 - 30% in the urine and 30 - 35% associated with the tissues.

MUTAGENICITY STUDIES

1. A Mutagenicity Test on Alkyl Dimethyl Benzyl Ammonium Chloride (ADBAC) in the CHO/HGPRT Forward Mutation Assay

Performing Laboratory: Hazleton Laboratories America, Inc.
Final Report Issued: 01/23/89
Purpose: EPA registration (Guideline Reference No. 84-4)
Results: No evidence of mutagenicity was noted with or without metabolic activation.

2. A Mutagenicity Test on Alkyl Dimethyl Benzyl Ammonium Chloride (ADBAC) in the Rat Primary Hepatocyte Unscheduled DNA Synthesis Assay

Performing Laboratory: Hazleton Laboratories America, Inc.
Final Report Issued: 01/25/89
Purpose: EPA registration (Guideline Reference No. 84-4)
Results: There was no evidence of an increase in unscheduled DNA synthesis.

3. Genotoxicity Test on Alkyl Dimethyl Benzyl Ammonium Chloride (ADBAC) in the Assay for Unscheduled DNA Synthesis in Rat Liver Primary Cell Cultures - Independent Repeat

Performing Laboratory: Hazleton Laboratories America, Inc.
Final Report Issued: 04/15/92
Purpose: EPA registration (Guideline Reference No. 84-4). (EPA requested an independent repeat of the previous UDS assay)
Results: There was no evidence of increased UDS activity, confirming results from the previous assay.

ECOTOXICOLOGY STUDIES

1. Evaluation of Alkyl Dimethyl Benzyl Ammonium Chloride (ADBAC) in a Static Renewal Acute Toxicity Test with Bluegill Sunfish (*Lepomis macrochirus*)

Performing Laboratory: Battelle
Study Duration: 96 hours
Final Report Issued: 06/26/91
Purpose: EPA registration (Guideline Reference No. 72-1(a))
Results: The 96-hour LC_{50} was determined to be 515 $\mu\text{g/L}$.

2. Evaluation of Alkyl Dimethyl Benzyl Ammonium Chloride (ADBAC) in a Static Renewal Acute Toxicity Test with Rainbow Trout (*Oncorhynchus mykiss*)

Performing Laboratory: Battelle
Study Duration: 96 hours
Final Report Issued: 06/26/91
Purpose: EPA registration (Guideline Reference No. 72-1(c))
Results: The 96-hour LC_{50} was determined to be 930 $\mu\text{g/L}$.

3. Evaluation of Alkyl Dimethyl Benzyl Ammonium Chloride (ADBAC) in a Static Acute Toxicity Test with Daphnids (*Daphnia magna*)

Performing Laboratory: Battelle
Study Duration: 48 hours
Final Report Issued: 06/26/91
Purpose: EPA registration (Guideline Reference No. 72-2(a))
Results: The 48-hour LC_{50} was determined to be 5.8 $\mu\text{g/L}$.

Summary of Completed Studies on ADBAC Quat

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4. A 96-Hour Static-Renewal Acute Toxicity Test with Alkyl Dimethyl Benzyl Ammonium Chloride (ADBAC) in the Sheepshead Minnow (*Cyprinodon variegatus*)

Performing Laboratory: Wildlife International, Ltd.
Final Report Issued: 09/15/92
Concentrations: 0, 0.42, 0.68, 1.1, 1.8, 2.8 ppm
Purpose: EPA registration (Guideline Reference No. 72-3 (a))
Results: The 96-Hour LC_{50} was determined to be 0.86 ppm.

5. A 48-Hour Static Acute Toxicity Test with Alkyl Dimethyl Benzyl Ammonium Chloride (ADBAC) in Embryo Larvae of the Eastern Oyster (*Crassostrea virginica*)

Performing Laboratory: Wildlife International, Ltd.
Final Report Issued: 09/15/92
Concentrations: 0, 25.0, 40.8, 58.6, 89.7, 145.0 ppb
Purpose: EPA registration (Guideline Reference No. 72-3 (b))
Results: The 48-Hour LC_{50} and EC_{50} were determined to be 55.2 and 47.6 ppb, respectively.

6. A 96-Hour Static-Renewal Acute Toxicity Test with Alkyl Dimethyl Benzyl Ammonium Chloride (ADBAC) in the Saltwater Mysid (*Mysidopsis bahia*)

Performing Laboratory: Wildlife International, Ltd.
Final Report Issued: 09/15/92
Concentration Evaluated: 0, 0.030, 0.047, 0.081, 0.13, 0.22, 0.35 ppm
Purpose: EPA registration (Guideline Reference No. 72-3 (c))
Results: The 96-Hour LC_{50} was determined to be 0.092 ppm.

7. Daily Static-Renewal Early Life Stage Toxicity Test of Alkyl Dimethyl Benzyl Ammonium Chloride (ADBAC) to Fathead Minnows

Performing Laboratory: Battelle
Final Report Issued: 03/30/92
Concentration Evaluated: 0.0, 27, 74, 135, 180, 270, 490 $\mu\text{g/L}$
Duration: 34-day total, egg plus 28-day post-hatch exposures
Purpose: EPA registration (Guideline Reference No. 72-4(a))
Results: Clear effects ($\geq 488.7 \mu\text{g/L}$) and no effects ($273.2 \mu\text{g/L}$) on hatchability were observed. The 28-day post-hatch LC_{50} was determined to be 94 $\mu\text{g/L}$ and no-effect levels for survival (32.2 $\mu\text{g/L}$) and growth ($\geq 32.2 \mu\text{g/L}$) were determined.

Summary of Completed Studies on ADBAC Quat

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8. Static-Renewal Chronic 21-Day Toxicity Test of Alkyl Dimethyl Benzyl Ammonium Chloride (ADBAC) to *Daphnia magna*

Performing Laboratory: Battelle
Final Report Issued: 03/30/92
Concentration Evaluated: 0.0, 0.5, 0.9, 1.6, 2.8, 5.0 µg/L
Purpose: EPA registration (Guideline Reference No. 72-4(b))
Results: Clear effect on reproduction (≥ 5.02 µg/L) was determined in the range-finding study and no effects on survival, growth, or reproduction (≤ 4.15 µg/L) were observed in the definitive study.

9. Static-Renewal Acute Toxicity of ADBAC to Fathead Minnow (*Pimephales promelas*) in Dilution Water With 0, 10 and 20 mg/L Humic Acid (3 studies)

Performing Laboratory: Analytical Bio-Chemistry Laboratories, Inc.
Final Reports Issued: 10/31/94, 11/02/94 and 10/31/94, respectively
Purpose: To determine whether the addition of naturally occurring dissolved organics would ameliorate the acute lethal effect (LC_{50}) of ADBAC to fathead minnows
Results: The 96-hour LC_{50} s for ADBAC in clean dilution water and in dilution water amended with 10 mg/ml and 20 mg/ml humic acid are 280, 800 and 1400 ppb, respectively.

*10. Chronic Toxicity of Sediment - Incorporated ADBAC to *Chironomus tentans* (Midge)

Performing Laboratory: Analytical Bio-Chemistry Laboratories, Inc.
Final Report Issued: 01/04/95
Purpose: To assess the chronic effects of sediment incorporated ADBAC on the midge
Results: The 28-day LC_{50} was determined to be 479 mg/kg, the 28-day NOEC was 520 mg/kg and the 28-day LOEC was 1200 mg/kg. Effects on growth and time to emergence were observed at levels ≥ 520 mg/kg and ≥ 1200 mg/kg, respectively.

ENVIRONMENTAL FATE STUDIES

1. Hydrolysis of ADBAC as a Function of pH at 25°C

Performing Laboratory: Analytical Bio-Chemistry Laboratories, Inc.
Study Duration: 30 days
Final Report Issued: 04/08/88
Concentration Evaluated: 10 µg/ml
Purpose: EPA registration (Guideline Reference No. 161-1)
Results: ADBAC was found to be stable hydrolytically in the pH range of 5 - 9 at 25°C.

2. Determination of the Photolysis Rate of ADBAC in pH 7 Buffered Solution at 25°C

Performing Laboratory: Analytical Bio-Chemistry Laboratories, Inc.
Study Duration: 30 days
Final Report Issued: 09/23/88
Concentration Evaluated: 10 µg/ml
Purpose: EPA registration (Guideline Reference No. 161-2)
Results: ADBAC was found to be photolytically stable in water at 25°C.

3. Anaerobic Aquatic Metabolism of Alkyl Dimethyl Benzyl Ammonium Chloride

Performing Laboratory: Analytical Bio-Chemistry Laboratories, Inc.
Study Duration: 1 year
Final Report Issued: 02/27/89
Concentration Evaluated: 10 ppm
Purpose: EPA registration (Guideline Reference No. 162-3)
Results: No degradation was observed.

4. Aerobic Aquatic Metabolism of Alkyl Dimethyl Benzyl Ammonium Chloride

Performing Laboratory: Analytical Bio-Chemistry Laboratories, Inc.
Study Duration: 30 days
Final Report Issued: 07/27/88
Concentration Evaluated: 10 µg/ml
Purpose: EPA registration (Guideline Reference No. 162-4)
Results: No degradation was observed.

5. Soil/Sediment Adsorption-Desorption of Alkyl Dimethyl Benzyl Ammonium Chloride

Performing Laboratory: Analytical Bio-Chemistry Laboratories, Inc
Study Duration: 30 days
Final Report Issued: 07/27/88
Concentrations Evaluated: 0.0, 0.1, 0.5, 1.0, 2.0 µg/ml
Soil Types: #32 sand, #45 silt loam, #59 sandy loam,
#58 clay loam
Purpose: EPA registration (Guideline Reference No. 163-1)
Results: ADBAC was found to be immobile in all four types.

6. Bioconcentration and Elimination of ¹⁴C-Residues by Bluegill (*Lepomis macrochirus*)
Exposed to Alkyl Dimethyl Benzyl Ammonium Chloride (ADBAC)

Performing Laboratory: Springborn Life Sciences, Inc.
Study Duration: 8 weeks
Final Report Issued: 03/06/89
Concentration Evaluated: 0.050 ppm
Purpose: EPA registration (Guideline Reference No. 165-4)
Results: The bioconcentration factor in nonedible tissue was 60 x
and in edible tissue was 33 x. These are considered to be
relatively low bioconcentration factors and indicate that
ADBAC would not expect to bioaccumulate in fish tissue.

BIODEGRADATION STUDIES

1. Assessment of the Biodegradability of ADBAC by Roy F. Weston, Inc.

The open literature was reviewed by Roy F. Weston, Inc. to obtain available information on the biodegradation of ADBAC and similar quaternary ammonium compounds. The weight of the evidence indicates that ADBAC is biodegradable and environmentally acceptable.

2. Determination of the Acute Toxicity of Chemicals and Wastewaters to Aquatic Microorganisms - Hyamine 3500-80

Performing Laboratory: Roy F. Weston, Inc.
Final Report Issued: 12/10/92
Purpose: To determine the level at which ADBAC is toxic to microbes in activated sludge
Results: ADBAC was found to be toxic at concentrations > 10 mg/L for unacclimated microbes and > 20 mg/L for acclimated microbes.

3. Semi-Continuous Activated Sludge (SCAS) Removability Test - Hyamine 3500-80

Performing Laboratory: Roy F. Weston, Inc.
Final Report Issued: 12/10/92
Purpose: To determine biodegradability by measuring the removal of soluble organic carbon
Results: Essentially 100% of all soluble organic carbon removed.

4. CO₂ Production Test - Hyamine 3500-80

Performing Laboratory: Roy F. Weston, Inc.
Final Report Issued: 12/10/92
Purpose: To determine biodegradability by measuring the amount of CO₂ produced by microorganisms in activated sludge when ADBAC was the sole carbon source
Results: Ultimate biodegradation comparable to the reference compound d-glucose was observed.

MISCELLANEOUS STUDIES

1. Leaching Study of Alkyl Dimethyl Benzyl Ammonium Chloride (ADBAC) Under Simulated Use Conditions

Performing Laboratory: Biological Test Center
Final Report Issued: 12/19/88
Study Design: Patches treated with ADBAC at a concentration of 300 ppm were applied to the shaved, moistened backs of guinea pigs for 6 hours per day for 5 consecutive days
Purpose: EPA required definition of the amount of leaching from ADBAC treated fabric under simulated use conditions
Results: Approximately 45% of the ADBAC leached out of the treated fabric.

2. Alkyl Dimethyl Benzyl Ammonium Chloride (ADBAC) Volatility Study

Performing Laboratory: Bushy Run Research Center
Final Report Issued: 05/22/90
Study Design: Air was passed over the surface of a 50 ppm ADBAC solution for 24 hours
Purpose: To determine the potential for ADBAC to volatilize from a system simulating that of a portable home humidifier
Results: No volatility was observed.

TRS

Toxicology/Regulatory Services

SUMMARY OF COMPLETED STUDIES ON DDAC¹

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MAMMALIAN TOXICOLOGY STUDIES

1. Acute Oral Toxicity in Rats - Median Lethal Dosage Determination with Didecyltrimethylammoniumchloride (DDAC)

Performing Laboratory: Hill Top Biolabs, Inc.
Study Initiation: 06/03/91
Final Report Issued: 04/09/92
Purpose: EPA registration (Guideline Reference No. 81-1)
Results: The LD₅₀ was 0.238 g/kg. The NOEL was 0.01 g/kg.

2. Primary Eye Irritation Study in Rabbits of Didecyltrimethylammoniumchloride (DDAC)

Performing Laboratory: Hill Top Biolabs, Inc.
Study Initiation: 06/03/91
Final Report Issued: 10/03/91
Study Design: Single animal screen
Purpose: EPA registration (Guideline Reference No. 81-4)
Results: Severe eye irritation and corrosion were observed.

3. Primary Skin Irritation Study in Rabbits of Didecyltrimethylammoniumchloride (DDAC)

Performing Laboratory: Hill Top Biolabs, Inc.
Study Initiation: 06/03/91
Final Report Issued: 10/03/91
Study Design: Single animal screen
Purpose: EPA registration (Guideline Reference No. 81-5)
Results: Severe dermal irritation and corrosion were observed.

¹ No new studies have been added since last summary (04/94).

4. Photoallergy Study in Guinea Pigs of Didecyldimethylammoniumchloride (DDAC)

Performing Laboratory: Hill Top Biolabs, Inc.
Study Initiation: 01/03/91
Final Report Issued: 10/03/91
Study Design: Buehler method at 0.1%
Purpose: EPA registration (Guideline Reference No. 81-6)
Results: There was no evidence of photoallergy or contact sensitization.

5. Two-Week Skin Irritation Study with Didecyldimethylammoniumchloride in Rats

Performing Laboratory: Bushy Run Research Center
Study Initiation: 11/02/87
Final Report Issued: 11/30/88
Treatment Concentrations: 0, 0.03, 0.1, 0.3, 0.6, 1.0, 3.0%
Purpose: To obtain data from which to select dosage levels for a definitive 90-day dermal toxicity study
Results: The highest concentration was defined at which only slight irritation was observed, i.e. (0.6%). No systemic toxicity was observed at any level.

6. Ninety-Day Subchronic Dermal Toxicity Study with Didecyldimethylammoniumchloride in Rats

Performing Laboratory: Bushy Run Research Center
Study Initiation: 01/11/88
Final Report Issued: 10/07/88
Treatment Concentrations/
Dosage Levels: 0, 0.1, 0.3, 0.6% (w/w) corresponding to dosage levels of 0, 2, 6, 12 mg/kg b.w./day, respectively
Purpose: EPA registration (Guideline Reference No. 82-3)
Results: Slight skin irritation was noted in all treatment groups. No systemic toxic effects were observed at any dosage level. Highest concentration represents the maximum dose of DDAC which could be evaluated in this test system.

7. Short Term Palatability Study of Didecyldimethylammoniumchloride in Dogs

Performing Laboratory: Hazleton Laboratories America, Inc.
Study Initiation: 04/20/89
Final Report Issued: 04/24/90
Dosage Levels: 0, 30, 60, 90 mg/kg/day
Purpose: To evaluate the palatability of DDAC in the diet to dogs
Results: Dietary rejection was observed at all dose levels.

8. Subchronic Oral Toxicity Study of Didecyldimethylammoniumchloride in Dogs

Performing Laboratory: Hazleton Laboratories America, Inc.
Study Initiation: 11/15/88
Final Report Issued: 04/24/90
Dosage Levels/Duration: 0, 7.5, 15, 30, 60 mg/kg/day by oral gavage for eight weeks.
Because of frequent emesis in the three highest groups, the single daily dosing regimen was changed to two equally divided (same total dose) a.m. and p.m. doses starting in week three and continuing until termination.
Purpose: To evaluate the subchronic toxicity of DDAC when administered daily by oral gavage to dogs for eight weeks
Results: Numerous treatment-related effects, including emesis, clinical signs and mortality, were observed at the 60 mg/kg/day level. Similar but less severe effects were observed in the 30 mg/kg/day dose group. Effects at the 15 mg/kg/day level were limited to emesis and soft stools. The severity of effects in all groups was reduced by the twice a day dosing regimen. The only treatment-related effect in the 7.5 mg/kg/day group was soft mucoid feces.

9. Chronic Oral Toxicity Study of Didecyldimethylammoniumchloride in Dogs

Performing Laboratory: Hazleton Washington
Study Initiation: 06/06/89
Final Report Issued: 07/26/91
Dosage Levels: 0, 3, 10, 30/20 mg/kg/day. Dosing at the 30 mg/kg/day level was discontinued on day 31 and reinstated at 20 mg/kg/day on day 36.
Purpose: EPA registration (Guideline Reference No. 83-1(b))
Results: Clear effect (≥ 20 mg/kg/day) and no-effect (≤ 10 mg/kg/day) levels were determined for systemic toxicity. Treatment did not have any effect on the incidence of tumors.

10. Ninety-Day Dietary Dose Range-Finding Study with Didecyldimethylammoniumchloride in Mice

Performing Laboratory: Bushy Run Research Center
Study Initiation: 09/17/87
Final Report Issued: 09/12/88
Dietary Concentrations: 0, 100, 300, 600, 1000, 3000 ppm
Purpose: To obtain data from which to select dosage levels for long term studies
Results: Clear effect (≥ 1000 ppm) and no-effect (≤ 600 ppm) levels were determined. Dose response was found to be very steep. Further elucidation of dose response was needed between the 1000 and 3000 ppm dosage concentrations.

11. Two-Week Dose Range-Finding Study with Didecyldimethylammoniumchloride in Mice

Performing Laboratory: Bushy Run Research Center
Study Initiation: 05/07/88
Final Report Issued: 12/01/88
Dietary Concentrations: 0, 1500, 2000 ppm
Purpose: To further elucidate dose response between the 1000 and 3000 ppm concentrations
Results: Gradation of effects between 1000 and 3000 ppm were defined.

Summary of Completed Studies - DDAC

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12. Chronic Dietary Oncogenicity Study with Didecyldimethylammoniumchloride in Mice

Performing Laboratory: Bushy Run Research Center
Study Initiation: 07/05/88
Final Report Issued: 02/07/91
Dietary Concentrations: 0, 100, 500, 1000 ppm
Purpose: EPA registration (Guideline Reference No. 83-2)
Results: Clear effect (1000 ppm) and no-effect (≤ 500 ppm) levels were determined. Treatment did not have any effect on survival or tumor incidences.

13. Developmental Toxicity Dose Range-Finding Study of Didecyldimethylammoniumchloride Administered by Gavage to CD* (Sprague-Dawley) Rats

Performing Laboratory: Bushy Run Research Center
Study Initiation: 12/05/89
Final Report Issued: 02/26/91
Dosage Levels: 0.0, 1.0, 12.5, 25.0, 37.5, 50.0 mg/kg/day
Purpose: To obtain data from which to select dose levels for a definitive teratology study in rats
Results: Maternal toxicity was observed at dosage levels ≥ 12.5 mg/kg/day. Treatment had no effect of gestational parameters or on the incidence of external variations or malformations.

14. Developmental Toxicity Evaluation of Didecyldimethylammoniumchloride Administered by Gavage to CD* (Sprague-Dawley) Rats

Performing Laboratory: Bushy Run Research Center
Study Initiation: 02/12/90
Final Report Issued: 05/17/91
Dosage Levels: 0, 1, 10, 20 mg/kg/day
Purpose: EPA registration (Guideline Reference No. 83-3(a))
Results: Clear effect (≥ 10 mg/kg/day) and no-effect (1 mg/kg/day) levels were determined for maternal toxicity. Treatment did not have any effect on the gestational parameters or incidences of variations or malformations.

15. Didecyldimethylammoniumchloride Acute Peroral Toxicity Study in the Female Rabbit

Performing Laboratory: Bushy Run Research Center
Study Initiation: Fourth quarter, 1987
Final Report Issued: 09/14/88
Dosage Levels: 50, 100, 200 mg/kg
Purpose: To obtain data from which to select dosage levels for a dose range-finding teratology study in rabbits
Results: Treatment-related mortality occurred at the high-dose level.

16. Developmental Toxicity Dose Range-Finding Study of Didecyldimethylammoniumchloride Administered by Gavage to New Zealand White Rabbits

Performing Laboratory: Bushy Run Research Center
Study Initiation: 01/24/88
Final Report Issued: 10/31/88
Dosage Levels: 0, 1, 3, 10, 30, 100 mg/kg/day
Purpose: To obtain data from which to select dosage levels for a definitive teratology study in rabbits
Results: Maternal toxicity, including mortality, occurred at dosage levels ≥ 30 mg/kg/day. Clinical signs and reduced food consumption were observed at dosage levels ≥ 3 mg/kg/day. The no-effect level for maternal toxicity was 1.0 mg/kg/day. Treatment had no effect on the gestational parameters or the incidences of variations or malformations.

17. Developmental Toxicity Study of Didecyldimethylammoniumchloride Administered by Gavage to New Zealand White Rabbits

Performing Laboratory: Bushy Run Research Center
Study Initiation: 06/06/88
Final Report Issued: 01/27/89
Dosage Levels: 0, 1, 3, 10 mg/kg/day
Purpose: EPA registration (Guideline Reference No. 83-3(b))
Results: Clear effect (≥ 3 mg/kg/day) and no-effect (1 mg/kg/day) levels were determined for maternal toxicity. Treatment had no effect on the gestational parameters or incidences of variations or malformations.

Summary of Completed Studies - DDAC

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18. Ninety-Day Dietary Subchronic Oral Toxicity Study with
Didecyldimethylammoniumchloride in Rats

Performing Laboratory: Bushy Run Research Center
Study Initiation: 09/16/87
Final Report Issued: 09/06/88
Dietary Concentrations: 0, 100, 300, 600, 1000, 3000 ppm
Purpose: To obtain data from which to select dosage levels for long term studies
Results: Clear effect (3000 ppm) and no-effect (≤ 1000 ppm) levels were determined. Dose response was found to be very steep. Further elucidation of dose response was needed between the 1000 and 3000 ppm levels.

19. Two-Week Dose Range-Finding Study with Didecyldimethylammoniumchloride in Rats

Performing Laboratory: Bushy Run Research Center
Study Initiation: 04/18/88
Final Report Issued: 12/01/88
Dietary Concentrations: 0, 1500, 2000 ppm
Purpose: To further elucidate dose response between the 1000 and 3000 ppm concentrations
Results: Gradation of effects between 1000 and 3000 ppm were defined.

20. Two-Generation Reproduction Study in (CD*) Sprague-Dawley Rats with
Didecyldimethylammoniumchloride Administered in the Diet

Performing Laboratory: Bushy Run Research Center
Study Initiation: 05/31/88
Final Report Issued: 02/01/91
Dietary Concentrations: 0, 300, 750, 1500 ppm
Purpose: EPA registration (Guideline Reference No. 83-4)
Results: Clear effect (1500 ppm) and no-effect (≤ 750 ppm) levels were determined for parental and postnatal toxicity. There were no effects on any of the reproductive parameters.

Summary of Completed Studies - DDAC

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21. Chronic Dietary Toxicity/Oncogenicity Study with Didecyldimethylammoniumchloride in Rats

Performing Laboratory: Bushy Run Research Center
Study Initiation: 06/13/88
Final Report Issued: 06/27/91
Dietary Concentrations: 0, 300, 750, 1500 ppm
Purpose: EPA registration (Guideline Reference No. 83-5)
Results: Clear effect (1500 ppm) and no-effect (≤ 750 ppm) levels were determined. Treatment had no effect on survival or the incidence of tumors.

22. Absorption, Distribution, Metabolism and Excretion Studies of Didecyldimethylammoniumchloride (DDAC) in the Rat

Performing Laboratory: Biological Test Center
Study Initiation: 12/15/87
Final Report Issued: 12/01/89
Study Design: The following 3 regimens were evaluated:
1. single low-dose oral (5 mg/kg)
2. repeated low-dose oral (5 mg/kg)
3. single high-dose oral (50 mg/kg)
Purpose: EPA registration (Guideline Reference No. 85-1)
Results: 89 - 99% of DDAC was excreted in the feces, $< 2.5\%$ in the urine and $< 1.0\%$ was found in the tissues. The only metabolism which occurred involved oxidation of the two decyl side chains to hydroxy and hydroxyketo derivatives. Most, if not all, the metabolism appears to be microbial in nature.

MUTAGENICITY STUDIES

1. *Salmonella*/Mammalian Microsome Assay with Bardac 22

Performing Laboratory: Institute of Toxicology (Switzerland)
Study Initiation: Not reported
Final Report Issued: 07/16/82
Purpose: EPA registration (Guideline Reference No. 84-2(a))
Results: No evidence of mutagenicity was noted with or without metabolic activation.

2. P0151: Chromosomal Aberrations Assay with Chinese/Hamster Ovary Cells *in vitro*

Performing Laboratory: Inveresk Research International
Study Initiation: 08/26/86
Final Report Issued: 10/86
Purpose: EPA registration (Guideline Reference No. 84-2(b))
Results: No evidence of mutagenicity was noted with or without metabolic activation.

3 Mutagenicity Test on Didecyldimethylammoniumchloride (DDAC) in the CHO/HGPRT Forward Mutation Assay

Performing Laboratory: Hazleton Laboratories America, Inc.
Study Initiation: 12/14/87
Final Report Issued: 09/09/88
Purpose: EPA registration (Guideline Reference No. 84-4)
Results: No evidence of mutagenicity was noted with or without metabolic activation.

4 Mutagenicity Test On Didecyldimethylammoniumchloride (DDAC) in the Rat Primary Hepatocyte Unscheduled DNA Synthesis Assay

Performing Laboratory: Hazleton Laboratories America, Inc.
Study Initiation: 06/08/88
Final Report Issued: 09/12/88
Purpose: EPA registration (Guideline Reference No. 84-4)
Results: There was no evidence of an increase in unscheduled DNA synthesis.

ECOTOXICOLOGY STUDIES

1. Didecyldimethylammoniumchloride: An Acute Oral Toxicity Study with the Northern Bobwhite

Performing Laboratory: Wildlife International Ltd.
Study Initiation: 09/17/90
Final Report Issued: 01/14/91
Purpose: EPA registration (Guideline Reference No. 71-1)
Results: The LD₅₀ was 229 mg/kg. The no-effect level was < 31 mg/kg.

2. Didecyldimethylammoniumchloride: A Dietary LC₅₀ Study with the Northern Bobwhite

Performing Laboratory: Wildlife International Ltd.
Study Initiation: 08/16/90
Final Report Issued: 01/14/91
Concentrations: 0, 562, 1000, 1780, 3160, 5620 ppm
Purpose: EPA registration (Guideline Reference No. 71-2)
Results: The LC₅₀ was > 5620 ppm. The no-effect level was 1780 ppm.

3. Didecyldimethylammoniumchloride: A Dietary LC₅₀ Study with the Mallard

Performing Laboratory: Wildlife International Ltd.
Study Initiation: 08/16/90
Final Report Issued: 01/14/91
Concentrations: 0, 562, 1000, 1780, 3160, 5620 ppm
Purpose: EPA registration (Guideline Reference No. 71-2)
Results: The LC₅₀ was > 5620 ppm. The no-effect level was 562 ppm.

4. Evaluation of Didecyldimethylammoniumchloride (DDAC) in a Static Acute Toxicity Test with Bluegill Sunfish, *Lepomis macrochirus*

Performing Laboratory: Springborn Laboratories, Inc.
Study Initiation: 09/13/89
Study Duration: 96 hours
Final Report Issued: 07/10/90
Purpose: EPA registration (Guideline Reference No. 72-1(a))
Results: The 96-hour LC₅₀ was determined to be 320 µg A.I./L.

5. Evaluation of Didecyldimethylammoniumchloride (DDAC) in a Static Acute Toxicity Test with Coho Salmon *Oncorhynchus kisutch*

Performing Laboratory: Springborn Laboratories, Inc.
Study Initiation: 03/19/90
Study Duration: 96 hours
Final Report Issued: 07/11/90
Purpose: EPA registration (Guideline Reference No. 72-1)
Results: The 96-hour LC₅₀ was determined to be 1.0 mg A.I./L.

Summary of Completed Studies - DDAC

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6. Evaluation of Didecyldimethylammoniumchloride (DDAC) in a Static Acute Toxicity Test with Daphnids, *Daphnia magna*

Performing Laboratory: Springborn Laboratories, Inc.
Study Initiation: 09/21/89
Study Duration: 48 hours
Final Report Issued: 07/10/90
Purpose: EPA registration (Guideline Reference No. 72-2(a))
Results: The 48-hour EC_{50} was determined to be 94 $\mu\text{g A.I./L}$.

7. Evaluation of Didecyldimethylammoniumchloride (DDAC) in a Static Acute Toxicity Test with Mysid Shrimp, *Mysidopsis bahia*

Performing Laboratory: Springborn Laboratories, Inc.
Study Initiation: 01/23/90
Study Duration: 96 hours
Final Report Issued: 07/10/90
Purpose: EPA registration (Guideline Reference No. 72-3)
Results: The 96-hour LC_{50} was determined to be 69 $\mu\text{g A.I./L}$.

ENVIRONMENTAL FATE STUDIES

1. Hydrolysis of Didecyldimethylammoniumchloride (DDAC) as a Function of pH at 25°C

Performing Laboratory: Analytical Bio-Chemistry Laboratories
Study Initiation: 07/28/88
Study Duration: 30 days
Final Report Issued: 04/21/89
Concentration: 10 $\mu\text{g/ml}$
Purpose: EPA registration (Guideline Reference No. 161-1)
Results: DDAC was found to be stable hydrolytically in the pH range of 5 to 9 at 25°C.

Summary of Completed Studies - DDAC

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2. Determination of the Photolysis Rate of Didecyldimethylammoniumchloride (DDAC) in pH Buffered Solution at 25°C

Performing Laboratory: Analytical Bio-Chemistry Laboratories
Study Initiation: 08/10/88
Study Duration: 30 days
Final Report Issued: 04/24/89
Concentration: 10 µg/ml
Purpose: EPA registration (Guideline Reference No. 161-2)
Results: DDAC was found to be stable photolytically in water at 25°C.

3. Aerobic Soil Metabolism of ¹⁴C-Didecyldimethylammoniumchloride (¹⁴C-DDAC)

Performing Laboratory: Analytical Bio-Chemistry Laboratories
Study Initiation: 06/30/88
Study Duration: 1 year
Final Report Issued: 08/06/91
Concentration: 10 ppm
Purpose: EPA registration (Guideline Reference No. 162-1)
Results: DDAC was found to be stable with very little degradation during the year-long study. The half-life for degradation was determined to be 1,048 days.

4. Anaerobic Aquatic Metabolism of ¹⁴C-Didecyldimethylammoniumchloride (¹⁴C-DDAC)

Performing Laboratory: Analytical Bio-Chemistry Laboratories
Study Initiation: 10/20/88
Study Duration: 1 year
Final Report Issued: 08/06/91
Concentration: 10 ppm
Purpose: EPA registration (Guideline Reference No. 162-3)
Results: DDAC was stable and no degradation was observed during the year-long study.

Summary of Completed Studies - DDAC

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5. Aerobic Aquatic Metabolism of ^{14}C -Didecyldimethylammoniumchloride (^{14}C -DDAC)

Performing Laboratory: Analytical Bio-Chemistry Laboratories
Study Initiation: 06/30/88
Study Duration: 1 year
Final Report Issued: 08/06/91
Concentration: 10 ppm
Purpose: EPA registration (Guideline Reference No. 162-4)
Results: DDAC was stable and no degradation was observed during the year-long study.

6. Soil/Sediment Adsorption-Desorption of ^{14}C -Didecyldimethylammoniumchloride (DDAC)

Performing Laboratory: Analytical Bio-Chemistry Laboratories
Study Initiation: 03/13/89
Final Report Issued: 12/29/89
Concentration: 0.7, 3.5, 5.25, 7.0 ppm
Soil Types: Sand, sandy loam, silty clay loam, silt loam
Purpose: EPA registration (Guideline Reference No. 163-1)
Results: DDAC was found to be immobile in all four soil types.

7. Determination of the Photolysis Rate of Didecyldimethylammoniumchloride on the Surface of Soil

Performing Laboratory: Analytical Bio-Chemistry Laboratories
Study Initiation: 06/13/91
Study Duration: 30 days
Final Report Issued: 09/11/92
Purpose: EPA Registration (Guideline Reference No. 161-3)
Results: DDAC was found to be stable photolytically in soil at 25°C.

8. Bioconcentration and Elimination of ^{14}C -Residues by Bluegill (*Lepomis macrochirus*)
Exposed to Didecyldimethylammoniumchloride (DDAC)

Performing Laboratory:	Springborn Laboratories, Inc.
Study Initiation:	04/10/89
Study Duration:	28 day exposure/18 day depuration
Final Report Issued:	02/06/90
Concentration:	59 $\mu\text{g/L}$
Purpose:	EPA registration (Guideline Reference No. 165-4)
Results:	The bioconcentration factor in nonedible tissue was 140 x and in edible tissue was 38 x. These are considered to be relatively low bio-concentration factors and indicate that DDAC would not be expected to bioaccumulate in fish tissue.

MISCELLANEOUS STUDIES

Assessment of the Biodegradability of DDAC by Roy F. Weston, Inc.

The open literature was reviewed by Roy F. Weston, Inc. to obtain available information on the biodegradation of DDAC and similar quaternary ammonium compounds. The weight of the evidence indicates that DDAC is biodegradable and environmentally acceptable.

NTP TECHNICAL REPORT
ON THE
TOXICOLOGY AND CARCINOGENESIS
STUDIES OF
BENZETHONIUM CHLORIDE
(CAS NO. 121-54-0)
IN F344/N RATS AND B6C3F₁ MICE
(DERMAL STUDIES)

Scheduled Peer Review Date: June 21-22, 1994

NOTICE

This is a DRAFT Technical Report prepared for public review and comment. Until this DRAFT has been reviewed and approved by the NTP Board of Scientific Counselors' Technical Reports Review Subcommittee in public session, the interpretations described herein do not represent the official scientific position of the National Toxicology Program. Following peer review, readers should contact NTP for the final version of this Technical Report.

NTP TR 438

NHII Publication No. 94-3169

U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES
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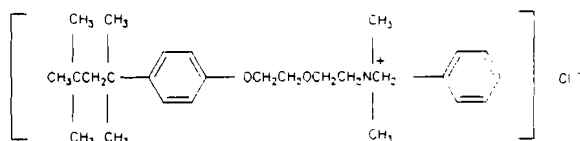
Prepared Technical Report

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ABSTRACT



BENZETHONIUM CHLORIDE

CAS No. 121-54-0

Chemical Formula: $C_{27}H_{42}NO_2 \cdot Cl$

Molecular Weight: 448.1

Synonyms: Benzylidimethyl-*p*-(1,1,3,3-tetramethylbutyl) phenoxyethoxy-ethylammonium chloride; diisobutylphenoxyethoxy-ethylidimethyl benzyl ammonium chloride; *p*-tert-octylphenoxyethoxyethylidimethylbenzyl ammonium chloride

Trade names: Anti-germ 77, Antiseptol, BZT, Diapp, Disilyn, Hyamine, Hyamine 1622, Phemeride, Phemithyn, Polymine D, Quatrachlor, Solamine

Benzethonium chloride is used primarily in cosmetics for its antimicrobial and cationic surfactant properties. Benzethonium chloride was nominated by the National Cancer Institute to the NTP for study from a class study of chemicals used as biocides. The chemical was selected based on a suspicion of carcinogenicity and its known widespread human exposure. Male and female F344/N rats and B6C3F₁ mice were topically administered benzethonium chloride (greater than 98% pure) for 16 days, 13 weeks, or 2 years. Genetic toxicology studies were conducted in *Salmonella typhimurium* and cultured Chinese hamster ovary cells.

16-DAY STUDY IN RATS

Groups of five male and five female F344/N rats were topically administered 0, 6.3, 12.5, 25, 50, or 100 mg benzethonium chloride/kg body weight. Rats were administered a total of 16 doses in a fixed volume of 250 μ L ethanol. All rats survived to the end of the study. The final mean body weights and body weight gains of rats administered 50 or 100 mg benzethonium chloride/kg body weight were significantly less than those of the controls. Clinical findings at necropsy included thickening or hardening of the skin at the site

of application in all rats administered 50 or 100 mg/kg and in 25 mg/kg males. Lesions at the site of application appeared crusty or red-grey in color. Epithelial hyperplasia with or without inflammation occurred at the site of application in all groups of males and females administered benzethonium chloride.

16-DAY STUDY IN MICE

Groups of five male and five female B6C3F₁ mice were topically administered 0, 6.3, 12.5, 25, 50, or 100 mg benzethonium chloride/kg body weight. Mice were administered a total of 12 doses in a fixed volume of 100 μ L ethanol. One 100 mg/kg male mouse died on day 4 of the study. Final mean body weights of all groups of males and females were similar to those of the controls. Clinical findings included mild irritation at the site of application in 50 and 100 mg/kg males and females and in 25 mg/kg males. Epithelial hyperplasia with or without inflammation occurred at the site of application in all groups of males and females administered benzethonium chloride.

13-WEEK STUDY IN RATS

Groups of 10 male and 10 female F344/N rats were topically administered 0, 1.563, 3.125, 6.25, 12.5, or 25 mg benzethonium chloride/kg body weight, 5 days per week for 13 weeks. Doses were administered in ethanol at a volume not exceeding 300 μ L. All rats survived to the end of the study. The final mean body weight and body weight gain of 25 mg/kg males were significantly lower than those of the controls. The final mean body weights of all other groups of males and of all groups of females were similar to those of the controls. Clinical findings included irritation at the site of application in groups administered 3.125 mg/kg or greater. There were no differences in absolute or relative organ weights considered to be related to chemical administration. Epithelial hyperplasia was observed at the site of application in all groups of males and females administered benzethonium chloride. Additionally, inflammation and ulceration were observed at the site of application in males and females administered 3.125 mg/kg or

greater. Based on the lesions observed in the 13-week study, benzethonium chloride dose levels selected for the 2-year dermal study in male and female rats were 0.15, 0.5, and 1.5 mg/kg.

13-WEEK STUDY IN MICE

Groups of 10 male and 10 female B6C3F₁ mice were topically administered 0, 1.563, 3.125, 6.25, 12.5, or 25 mg benzethonium chloride/kg body weight, 5 days per week for 13 weeks. Doses were administered in ethanol at a volume not exceeding 100 μ L. All mice survived to the end of the study. The final mean body weights of all dosed groups of males and females were similar to those of the controls; the mean body weight gain of 25 mg/kg males was significantly less than that of the controls. Males administered 6.25, 12.5, or 25 mg benzethonium chloride/kg body weight developed irritation, thickening of the skin, scales, and/or discoloration at the site of application, as did female mice administered 12.5 or 25 mg/kg. Increased incidences of epithelial hyperplasia and inflammation were observed at the site of application in all groups of males and females administered benzethonium chloride. Based on the lesions observed in the 13-week study, benzethonium chloride dose levels selected for the 2-year dermal study in mice were 0.15, 0.5, and 1.5 mg/kg.

2-YEAR STUDY IN RATS

Groups of 60 male and 60 female F344/N rats were topically administered 0.15, 0.5, or 1.5 mg benzethonium chloride/kg body weight 5 days per week for 104 weeks. Doses were administered in ethanol, and dose volumes were adjusted weekly according to the average body weights of the groups. As many as nine rats per group were evaluated after 15 months of chemical administration.

Survival, Body Weights, and Clinical Findings

Survival of dosed rats was similar to that of the controls throughout the study. Mean body weights of all dosed groups of males and females were similar to those of the controls throughout the study. Reddening

of the skin was observed at the site of application in all dosed groups of males and females. There were no other clinical findings considered to be related to chemical administration.

Pathology Findings

There were no increased incidences of neoplasms in dosed male or female rats that were attributed directly to the administration of benzethonium chloride. Increased incidences of epithelial hyperplasia, sebaceous gland hyperplasia, and ulcers were observed at the site of application in dosed females. The incidence of epithelial hyperplasia was increased in 0.5 and 1.5 mg/kg males.

2-YEAR STUDY IN MICE

Groups of 60 male and 60 female B6C3F₁ mice were topically administered 0, 0.15, 0.5, or 1.5 mg benzethonium chloride/kg body weight 5 days per week for 104 weeks. Doses were administered in ethanol, and dose volumes were adjusted weekly according to the average body weights of the groups. As many as 10 mice per group were evaluated after 15 months of chemical administration.

Survival, Body Weights, and Clinical Findings

Survival of dosed mice was similar to that of the controls throughout the study. Mean body weights of all dosed groups of males and females were similar to those of the controls throughout the study. Reddening of the skin was observed at the site of application in all dosed groups of males and females. There were no other clinical findings attributed to chemical administration.

Pathology Findings

There were no increased incidences of neoplasms in dosed males or females that were related to administration of benzethonium chloride. Increased incidences of epithelial hyperplasia were observed at the site of application in dosed males and females.

GENETIC TOXICOLOGY

Benzethonium chloride was not mutagenic in *Salmonella typhimurium* strains TA98, TA100, TA1535, or TA1537, and did not induce sister chromatid exchanges or chromosomal aberrations in cultured Chinese hamster ovary cells. All tests were conducted with and without rat and hamster liver S9 metabolic activation enzymes.

CONCLUSIONS

Under the conditions of these 2-year dermal studies, there was *no evidence of carcinogenic activity** of benzethonium chloride in male or female F344/N rats receiving 0.15, 0.5, or 1.5 mg/kg. There was *no evidence of carcinogenic activity* in male or female B6C3F₁ mice receiving 0.15, 0.5, or 1.5 mg/kg.

Exposure of rats and mice to benzethonium chloride by dermal application in ethanol for 2 years resulted in epithelial hyperplasia in male and female rats and mice and sebaceous gland hyperplasia and ulcers in female rats at the site of application.

* Explanation of Levels of Evidence of Carcinogenic Activity is on page 11.

Summary of the 2-Year Carcinogenesis and Genetic Toxicology Studies of Benzethonium Chloride

Variable	Male F344/N Rats	Female F344/N Rats	Male B6C3F ₁ Mice	Female B6C3F ₁ Mice
Doses	0, 0.15, 0.5, or 1.5 mg/kg in not more than 296 μ L of acetone	0, 0.15, 0.5, or 1.5 mg/kg in not more than 317 μ L of acetone	0, 0.15, 0.5, or 1.5 mg/kg in not more than 131 μ L of acetone	0, 0.15, 0.5, or 1.5 mg/kg in not more than 131 μ L of acetone
Body weights	Dosed groups similar to controls	Dosed groups similar to controls	Dosed groups similar to controls	Dosed groups similar to controls
2-Year survival rates	15/52, 11/52, 9/54, 16/56	24/51, 33/53, 26/51, 24/53	43/50, 38/50, 42/50, 39/50	38/51, 34/53, 31/48, 34/54
Nonneoplastic effects	Skin (site of application): epithelial hyperplasia (1/52, 0/52, 4/55, 10/56)	Skin (site of application): epithelial hyperplasia (2/51, 2/53, 6/51, 32/53); sebaceous gland hyperplasia (1/51, 2/53, 6/51, 30/53); ulcer (0/51, 1/53, 3/51, 19/53)	Skin (site of application): epithelial hyperplasia (2/50, 7/50, 16/50, 23/50)	Skin (site of application): epithelial hyperplasia (3/52, 7/53, 6/48, 22/54)
Neoplastic effects	None	None	None	None
Level of evidence of carcinogenic activity	No evidence	No evidence	No evidence	No evidence
Genetic toxicology				
<i>Salmonella typhimurium</i> gene mutations:	Negative in strains TA98, TA100, TA1535, and TA1537 with and without S9			
Sister chromatid exchanges				
Cultured Chinese hamster ovary cells <i>in vitro</i> :	Negative with and without S9			
Chromosomal aberrations				
Cultured Chinese hamster ovary cells <i>in vitro</i> :	Negative with and without S9			

EXPLANATION OF LEVELS OF EVIDENCE OF CARCINOGENIC ACTIVITY

The National Toxicology Program describes the results of individual experiments on a chemical agent and notes the strength of the evidence for conclusions regarding each study. Negative results, in which the study animals do not have a greater incidence of neoplasia than control animals, do not necessarily mean that a chemical is not a carcinogen, inasmuch as the experiments are conducted under a limited set of conditions. Positive results demonstrate that a chemical is carcinogenic for laboratory animals under the conditions of the study and indicate that exposure to the chemical has the potential for hazard to humans. Other organizations, such as the International Agency for Research on Cancer, assign a strength of evidence for conclusions based on an examination of all available evidence, including animal studies such as those conducted by the NTP, epidemiologic studies, and estimates of exposure. Thus, the actual determination of risk to humans from chemicals found to be carcinogenic in laboratory animals requires a wider analysis that extends beyond the purview of these studies.

Five categories of evidence of carcinogenic activity are used in the Technical Report series to summarize the strength of the evidence observed in each experiment: two categories for positive results (**clear evidence** and **some evidence**); one category for uncertain findings (**equivocal evidence**); one category for no observable effects (**no evidence**); and one category for experiments that cannot be evaluated because of major flaws (**inadequate study**). These categories of interpretative conclusions were first adopted in June 1983 and then revised in March 1986 for use in the Technical Report series to incorporate more specifically the concept of actual weight of evidence of carcinogenic activity. For each separate experiment (male rats, female rats, male mice, female mice), one of the following five categories is selected to describe the findings. These categories refer to the strength of the experimental evidence and not to potency or mechanism.

- **Clear evidence** of carcinogenic activity is demonstrated by studies that are interpreted as showing a dose-related (i) increase of malignant neoplasms, (ii) increase of a combination of malignant and benign neoplasms, or (iii) marked increase of benign neoplasms if there is an indication from this or other studies of the ability of such tumors to progress to malignancy.
- **Some evidence** of carcinogenic activity is demonstrated by studies that are interpreted as showing a chemical-related increased incidence of neoplasms (malignant, benign, or combined) in which the strength of the response is less than that required for clear evidence.
- **Equivocal evidence** of carcinogenic activity is demonstrated by studies that are interpreted as showing a marginal increase of neoplasms that may be chemical related.
- **No evidence** of carcinogenic activity is demonstrated by studies that are interpreted as showing no chemical-related increases in malignant or benign neoplasms.
- **Inadequate study** of carcinogenic activity is demonstrated by studies that, because of major qualitative or quantitative limitations, cannot be interpreted as valid for showing either the presence or absence of carcinogenic activity.

When a conclusion statement for a particular experiment is selected, consideration must be given to key factors that would extend the actual boundary of an individual category of evidence. Such consideration should allow for incorporation of scientific experience and current understanding of long-term carcinogenesis studies in laboratory animals, especially for those evaluations that may be on the borderline between two adjacent levels. These considerations should include:

- adequacy of the experimental design and conduct;
- occurrence of common versus uncommon neoplasia;
- progression (or lack thereof) from benign to malignant neoplasia as well as from preneoplastic to neoplastic lesions;
- some benign neoplasms have the capacity to regress but others (of the same morphologic type) progress. At present, it is impossible to identify the difference. Therefore, where progression is known to be a possibility, the most prudent course is to assume that benign neoplasms of those types have the potential to become malignant;
- combining benign and malignant tumor incidence known or thought to represent stages of progression in the same organ or tissue;
- latency in tumor induction;
- multiplicity in site-specific neoplasia;
- metastases;
- supporting information from proliferative lesions (hyperplasia) in the same site of neoplasia or in other experiments (same lesion in another sex or species);
- presence or absence of dose relationships;
- statistical significance of the observed tumor increase;
- concurrent control tumor incidence as well as the historical control rate and variability for a specific neoplasm;
- survival-adjusted analyses and false positive or false negative concerns;
- structure-activity correlations; and
- in some cases, genetic toxicology.

NATIONAL TOXICOLOGY PROGRAM BOARD OF SCIENTIFIC COUNSELORS TECHNICAL REPORTS REVIEW SUBCOMMITTEE

The members of the Technical Reports Review Subcommittee who evaluated the draft NTP Technical Report on benzethonium chloride on June 21-22, 1994, are listed below. Subcommittee members serve as independent scientists, not as representatives of any institution, company, or governmental agency. In this capacity, subcommittee members have five major responsibilities in reviewing NTP studies:

- to ascertain that all relevant literature data have been adequately cited and interpreted.
- to determine if the design and conditions of the NTP studies were appropriate.
- to ensure that the Technical Report presents the experimental results and conclusions fully and clearly.
- to judge the significance of the experimental results by scientific criteria, and
- to assess the evaluation of the evidence of carcinogenic activity and other observed toxic responses.

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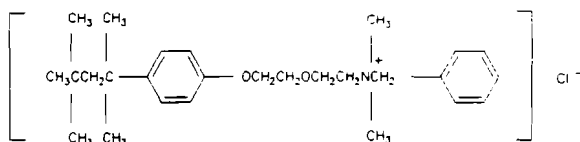
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SUMMARY OF TECHNICAL REPORTS REVIEW SUBCOMMITTEE COMMENTS

NOTE: A summary of the Technical Reports Review Subcommittee's remarks will appear in a future draft of this report.

INTRODUCTION



BENZETHONIUM CHLORIDE

CAS No. 121-54-0

Chemical Formula: $C_{27}H_{42}NO_2 \cdot Cl$

Molecular Weight: 448.1

Synonyms: Benzyl dimethyl-*p*-(1,1,3,3-tetramethylbutyl) phenoxyethoxy-ethyl ammonium chloride, diisobutylphenoxyethoxy-ethyl dimethyl benzyl ammonium chloride, *p*-tert-octylphenoxyethoxyethyl dimethyl benzyl ammonium chloride

Trade names: Anti-germ 77, Antiseptol, BZT, Diapp, Disilyn, Hyamine, Hyamine 1622, Phemeride, Phemithyn, Polymine D, Quatrachlor, Solamine

CHEMICAL AND PHYSICAL PROPERTIES

Benzethonium chloride is a white to colorless, nearly odorless, bitter tasting crystalline substance. It is soluble in water, alcohols, glycols, acetone, benzene, and other organic solvents (*Merck Index*, 1976; CTFA, 1985). Details of the synthesis of the compound are proprietary information. Commercial preparations typically contain numerous impurities including unreacted starting materials and side reaction products. Impurities may include benzyl chloride, benzal chloride, benzyl alcohol, benzaldehyde, and benzylamine derivatives (CIR, 1985).

PRODUCTION, USE, AND HUMAN EXPOSURE

Benzethonium chloride is used primarily in cosmetics for its antimicrobial and cationic surfactant properties. Concentrations below 1% (with most below 0.1%) are typically found in baby bath, eye makeup, personal hygiene, fragrance, hair, shaving, skin, and suntan preparations (CIR, 1985). The material is also widely used in disinfectants, cationic detergents, and preservatives, including uses in fabric

softening, ore flotation, corrosion inhibition, paper processing, and in pharmaceuticals and vaccines (CIR, 1985). Benzethonium chloride is used in a variety of over-the-counter drug products and is permitted at maximum concentrations of 0.01% in preparations for ophthalmic uses, and at 0.02% for other topical uses (FDA, 1980a). Mouth rinses containing benzethonium chloride at 0.075% to 0.1% have been shown to reduce plaque accumulation (Volpe *et al.*, 1969; Compton and Beagrie, 1975; Tanzer *et al.*, 1979; Gaffar *et al.*, 1980), but yellow-brown teeth and tongue discolorations are sometimes noted. Benzethonium chloride has also been incorporated into polymerized methyl methacrylate used in contact lenses (Mote *et al.*, 1969). Benzethonium chloride at 0.2% is spermicidal (Paniagua *et al.*, 1961).

Approximately 29,000 people are exposed occupationally to benzethonium chloride annually, and consumer exposure has been estimated to be approximately 3.8 million people per year in the U.S. (SRI, 1984).

Although quaternary ammonium compounds such as benzethonium chloride have been reported to have significant antimicrobial properties, their rather limited spectrum of action (primarily affecting gram positive bacteria) and a tendency to be inactivated by a large number of substances have caused their antimicrobial effectiveness to be questioned (FDA, 1980b). The primary germicidal action of benzethonium chloride has been attributed to an ability to disrupt cell membrane permeability (AMA, 1980). Concentrations of benzethonium chloride required to effect germicidal activity are reported to range from 0.005% to 0.01% (Christensen, 1963). Benzethonium chloride also has been shown to inhibit a number of proteolytic enzymes, and this inhibition can occur at concentrations below that needed to produce significant losses in bacterial viability (Stedman *et al.*, 1957; CIR, 1985). The compound also shows some inhibitory action on acetylcholinesterase (Jackson and Aprison, 1966).

ABSORPTION, DISPOSITION, METABOLISM, AND EXCRETION

Experimental Animals

La Rosa *et al.* (1978) reported that ^{14}C -labeled benzethonium chloride administered orally to pregnant rats on days 6 through 15 of gestation at doses of 1.125 and 3.558 mg/kg body weight per day resulted in increased maternal blood levels of radiolabel after each dosing. Accumulation in the fetuses was reported to be limited and variable. Insufficient information was given to more completely characterize the fate of the chemical.

The extent and rate of absorption following single and repeated dermal doses and the pattern of tissue distribution and route of elimination of [^{14}C] benzethonium chloride were studied in Fischer 344/N rats (NTP, 1988). The kinetics of distribution and excretion following an intravenous administration were also determined. From intravenous studies, an elimination half-life of radiolabel from blood was 110 minutes, the volume of distribution was 5.5 L/kg body weight, and the total clearance was 14.8 mL per minute per kg body weight. Twenty-four hours after a single intravenous dose, approximately 50% of the radiolabel was found in feces, and 4% in urine. In dermal studies, after application of 0.15 or 1.5 mg/kg ^{14}C -labeled compound to the skin under a non-occlusive patch, peak elimination of the radiolabel in urine and feces was observed at 24 to 48 hours (urine) or 48 to 72 hours (feces). Total urinary excretion was 1% to 2% of the applied dose, and fecal excretion accounted for about 45% of the dose. Radiolabel was below the detection limit in blood and most tissues during the study, but low levels were measured in the liver, and some residual radiolabel could be accounted for in the epidermis at the site of application. This could not be washed away. When similar studies were performed with repeated daily dermal dosing, the total amount of radiolabel excreted up to 10 days following the last dose was about 25%, suggesting some accumulation with repeated dermal administration. However, following these studies it was discovered that urinary excretion was likely underestimated due to adherence of benzethonium chloride to the glass walls of the metabolism cages (NTP, 1988).

Humans

There are no studies of the absorption, distribution, metabolism, or excretion of benzethonium chloride in humans.

TOXICITY

Experimental Animals

Acute oral LD₅₀ values for benzethonium chloride in rats (strain unspecified or Charles River CD) and in Charles River CD-1 mice range from 368 to 665 mg/kg (CIR, 1985). When benzethonium chloride was administered subcutaneously to Fischer 344/N rats, the LD₅₀ was reported as 119 mg/kg (Mason *et al.*, 1971), and intravenous administration resulted in LD₅₀ values of 19 mg/kg (rats) and 35 mg/kg (mice) in unspecified strains (CIR, 1985).

In a study designed to evaluate the ability of benzethonium chloride to inactivate influenza virus, co-administration of the virus with the chemical by intranasal instillation to mice was shown to reduce mortality due to the virus. Effective concentrations were in the range of 0.00625% to 0.0125%. Exposure of mice to 0.05 mL of 0.25% benzethonium chloride and greater doses were toxic, causing lobular consolidation, pneumonia, and death (Klein and Stevens, 1945).

Dermal administration of 0.1% benzethonium chloride to the clipped skin of rabbits caused no irritation or systemic toxicity over the course of a 4-week study in which a single daily dose was administered 5 days per week (Finnegan and Dienna, 1954). However, Homburger (1968) found severe local blistering and more moderate lesions at doses of 35 to 280 mg/kg benzethonium chloride (in tricapylin) following a single dermal application to the unshaved backs of C57BL/6 male mice. Benzethonium chloride is irritating to the rabbit eye at concentrations of 0.03% and greater (Finnegan and Dienna, 1954).

In studies of the primary irritancy and sensitization potential of benzethonium chloride in female B6C3F₁ mice, concentrations of greater than 10% (in 95% ethanol) were found to be irritating and 20% was selected as the challenge concentration to be used in the sensitization test. For this study, sensitization doses of 1%, 3%, and 10% were applied to the shaved and dermabraded dorsal surface daily for 5 days. Mice were challenged 7 days following the last treatment by application of 20% benzethonium chloride to the left ear. No evidence of contact hypersensitivity was noted in these studies (NTP, 1989).

Benzethonium chloride was administered by gavage to pregnant rats (strain not specified) on days 6 through 15 of gestation. At the highest dose of 35.6 mg per kg body weight per day, maternal weight gain was decreased, and delayed ossification was seen in the fetuses. The fetal effects were attributed to maternal toxicity (Gilman and De Salva, 1979).

Humans

Benzethonium chloride has been extensively studied for dermal irritant and sensitizing properties both alone and in various commercial formulations. Most results of these studies were negative, however case reports of contact sensitization have been described (CIR, 1985). Conjunctival reactions consisting of hyperemia, edema, capillary dilation, lacrimation, and desquamation of the conjunctival epithelium were frequently reported in tests of benzethonium chloride incorporated into ocular wetting solutions (Swan, 1944). Irritation is also a common reaction to vaginal aerosol foam contraceptives containing benzethonium (CIR, 1985).

Benzethonium chloride is cytotoxic to human cells. Several cultured human cell lines were reported killed by 10 µg/mL and 1 µg/mL inhibited their growth (Kuwahara *et al.*, 1976).

There are no other literature concerning the toxicity of benzethonium chloride in humans, nor is there any information concerning toxicity to the human immune, nervous, or reproductive systems.

Human data

CARCINOGENICITY

Experimental Animals

Benzethonium chloride has been evaluated for carcinogenicity in several species and by several routes of administration. The studies have been generally deficient in length of exposure time or in number of animals tested to be considered an adequate assessment of the carcinogenic potential of benzethonium chloride.

No gross or microscopic lesions attributed to chemical administration were observed when nine dogs were exposed to 5, 100, or 500 ppm benzethonium chloride in Purina dog chow for 1 year (Finnegan and Dienna, 1954). Purina dog chow mixed with 0, 50, 200, 1,000, 2,500, or 5,000 ppm benzethonium chloride was given to groups of 10 male and 10 female rats (strain unspecified) for 2 years (Finnegan and Dienna, 1954). Mortality was increased at 5,000 ppm, and thinning of the cecal wall and cecal distension at the three highest concentrations were the only abnormalities found.

Benzethonium chloride was administered by subcutaneous injection at doses of 0, 0.1, 0.3, 1.0, and 3.0 mg/kg to groups of 60, 10, 20, 30, or 40 Fischer 344/N male and female rats (respectively). Doses were administered twice per week for 1 year. The rats were kept an additional 6 months without dosing. Body weights were reduced by as much as 20% in the highest dose groups. Twenty-six of the 200 rats receiving benzethonium chloride developed sarcomas at the site of injection; no control rats developed sarcomas at the site of injection. The incidences of sarcomas were dose-related and were attributed to chemical administration. The neoplasms observed were described as typical fibrosarcomas arising from mesenchymal cells within an area of granulomatous reaction (Mason *et al.*, 1971).

Benzethonium chloride has been studied in a short-term mouse lung adenoma assay (Homburger, 1968). A single dose of 0.35 mg of the chemical was injected into the tail vein of 50 CF-1 and 50 A/Jax female mice. Twenty additional CF-1 female mice were administered monthly injections for 7 months, at which time all mice were evaluated. No increase in the incidence of pulmonary adenomas was found.

Humans

There were no studies found in the literature of the carcinogenicity of benzethonium chloride in humans.

GENETIC TOXICITY

Genotoxicity data for benzethonium chloride is limited to bacterial mutation and DNA damage tests. The chemical was not mutagenic, with or without Aroclor-induced S9 metabolic activation enzymes, in any of several strains of *Salmonella typhimurium* (DeFlora *et al.*, 1984a,b; Zeiger *et al.*, 1987). However, it was reported to induce DNA damage in several strains of repair-deficient *Escherichia coli* (DeFlora *et al.*, 1984b).

STUDY RATIONALE

Benzethonium chloride was nominated by the National Cancer Institute to the NTP for study from a class study of chemicals used as biocides (Johnson *et al.*, 1984). The chemical was selected based on a suspicion of carcinogenicity and its known widespread human exposure. The dermal route was recommended because of its predominant use in cosmetics and other topical products. The NTP has performed 16-day, 13-week, and 2-year studies in F344/N rats and B6C3F₁ mice by the dermal route (this report), and has studied the dermal absorption, distribution, and excretion of the chemical in the F344/N rat (NTP, 1988). Additional studies evaluated the potential irritant and contact hypersensitivity properties of benzethonium chloride in female B6C3F₁ mice (NTP, 1989), and the chemical's genetic toxicity in *Salmonella typhimurium* and cultured Chinese hamster ovary cells.

MATERIALS AND METHODS

PROCUREMENT AND CHARACTERIZATION OF BENZETHONIUM CHLORIDE

United States Pharmacopeia (USP) grade benzethonium chloride was obtained from Rohm and Haas (Philadelphia, PA) in one lot (W0061), which was used throughout the 16-day, 13-week, and 2-year dermal studies. Identity, purity, and stability analyses were conducted by the analytical chemistry laboratory, Midwest Research Institute (Kansas City, MO). Reports on analyses performed in support of the benzethonium chloride studies are on file at the National Institute of Environmental Health Sciences (NIEHS). The methods and results of these studies are detailed in Appendix G.

The chemical, a white powdered solid, was identified as benzethonium chloride by infrared, ultraviolet/visible, and nuclear magnetic resonance spectroscopy. The purity of the chemical was determined by elemental analyses, Karl Fischer water analysis, functional group titration, thin-layer chromatography, and high-performance liquid chromatography. Elemental analyses for carbon, hydrogen, nitrogen, and chlorine were in agreement with the theoretical values for benzethonium chloride. Functional group titration indicated a purity of $98.5\% \pm 0.5\%$. Karl Fischer water analysis indicated $0.6\% \pm 0.3(s)\%$ water. Thin-layer chromatography indicated a major spot and a trace impurity. High-performance liquid chromatography detected a major peak and no impurities greater than or equal to 0.1% of the major peak area. The overall purity was determined to be greater than 98%.

The complete battery of USP analyses was performed by the analytical chemistry laboratory as a supplement to the chemical characterization of benzethonium chloride. This lot of benzethonium chloride, was demonstrated to meet the specifications of all analyses required by the 20th revision of the USP.

Stability studies were performed by the analytical chemistry laboratory using high-performance liquid chromatography. These studies indicated that benzethonium chloride was stable as a bulk chemical for at

least 2 weeks when stored protected from light at temperatures up to 60° C. At the study laboratory, the bulk chemical was stored at room temperature protected from light. The stability of the chemical was monitored periodically using high-performance liquid chromatography. No degradation of the bulk chemical was observed.

PREPARATION AND ANALYSIS OF DOSE FORMULATIONS

Dose formulations were prepared once for the 16-day studies and every 2 weeks for the 13-week and 2-year studies by mixing benzethonium chloride and 95% ethanol (USP grade) to give the required concentrations (Table G1). Dose formulation stability studies were performed by the analytical chemistry laboratory using high-performance liquid chromatography. The stability of the benzethonium chloride dose formulation was confirmed for at least 3 weeks at room temperature when stored protected from light, and for at least 3 hours when exposed to light and air.

Periodic analyses of the dose formulations of benzethonium chloride sampled from the dose preparation laboratory as well as from the animal room were conducted by the study laboratory and the analytical chemistry laboratory using ultraviolet spectroscopy. During the 16-day studies, the dose formulations were analyzed once (Table G2); during the 13-week studies, the dose formulations were analyzed three times (Table G3); during the 2-year studies, the dose formulations were analyzed approximately every 2 months (Table G4). All of the dose formulations from the 16-day and 13-week studies were found to be within 10% of the target concentrations. In the 2-year study, 98% (169/173) of the dose formulations analyzed were within 10% of the target concentrations. Results of the periodic referee analyses performed by the analytical chemistry laboratory were in agreement with the results obtained by the study laboratory (Table G5).

16-DAY STUDIES

Male and female F344/N rats and B6C3F₁ mice were obtained from Simonsen Laboratories, Inc. (Gilroy, CA). On receipt, the rats and mice were 25 to 31 days old. Animals were quarantined for 11 days (rats) or 12 days (mice). Groups of five male and five female rats and mice were topically administered 0, 6.3, 12.5, 25, 50, or 100 mg benzethonium chloride per kg body weight. Doses were administered in ethanol at a fixed volume of 250 μ L (rats) or 100 μ L (mice). Doses were applied 5 days per week to the dorsal interscapular areas of the animals; the site of application was clipped three times during the studies. Animals were administered a total of 12 doses; animals were dosed for 2 (rats) or 3 (mice) consecutive days prior to necropsy and the last doses were applied less than 24 hours before necropsy. Feed and water were available *ad libitum*. Rats and mice were housed individually. Clinical findings were recorded on dosing days. The animals were weighed initially, on day 10, and at the end of the study. Details of the study design and animal maintenance are summarized in Table 1.

A necropsy was performed on all animals. The brain, heart, right kidney, liver, lung, right testis (rats), and thymus were weighed. For all animals, skin at the site of application and from undosed sites was examined histopathologically.

13-WEEK STUDIES

The 13-week studies were conducted to evaluate the cumulative toxic effects of repeated exposure to benzethonium chloride and to determine the appropriate doses to be used in the 2-year studies.

Male and female F344/N and B6C3F₁ mice were obtained from Simonsen Laboratories, Inc. (Gilroy, CA). On receipt, the rats were 25 to 31 days old and mice were 31 days old. Animals were quarantined for 27 days (mice) or 28 days (rats) before chemical administration began. Before the start of the studies, five male and five female rats and mice were selected for parasite evaluation and gross observation for evidence of disease. At the end of the studies, serologic analyses were performed on five male and five female control rats and mice using the protocols of the NTP Sentinel Animal Program (Appendix I).

Groups of 10 male and 10 female rats and mice were topically administered 0, 1.563, 3.125, 6.25, 12.5, or 25 mg benzethonium chloride per kg body weight. Doses were administered in ethanol and dose volumes were adjusted weekly according to the average body weights of the dosed groups. Dose volumes did not exceed 300 μL for rats or 100 μL for mice. Doses were applied 5 days per week to the dorsal interscapular areas of the animals; the site of application was clipped weekly during the studies. Feed and water were available *ad libitum*. Rats and mice were housed individually. Clinical findings were recorded weekly for rats and mice. The animals were weighed initially, weekly, and at the end of the studies. Details of the study design and animal maintenance are summarized in Table 1.

At the end of the 13-week studies, a necropsy was performed on all animals. The brain, heart, right kidney, liver, lungs, right testis, and thymus were weighed. Tissues for microscopic examination were fixed and preserved in 10% neutral buffered formalin, processed and trimmed, imbedded in paraffin, sectioned to a thickness of 5 to 6 μm , and stained with hematoxylin and eosin. A complete histopathologic evaluation was performed on all rats and mice in the control and 25 mg/kg groups. Table 1 lists the tissues and organs routinely examined. In addition, sections of treated and untreated skin from rats and mice in all dose groups were examined microscopically.

2-YEAR STUDIES

Study Design

Groups of 60 male and 60 female rats and mice were topically administered 0, 0.15, 0.5, or 1.5 mg benzethonium chloride per kg body weight. Doses were administered in ethanol and dose volumes were adjusted weekly according to the average body weights of the dosed groups. Throughout the study, dose volumes ranged from 63 to 296 μL (male rats), 95 to 317 μL (female rats), or 50 to 131 μL (mice). Doses were applied 5 days per week to the dorsal interscapular areas of the animals; the site of application was clipped approximately once per week during the studies. As many as 10 male and 10 female rats and mice were evaluated at 15 months for histopathology and organ weights.

Source and Specification of Animals

Male and female F344/N and B6C3F₁ mice were obtained from Taconic Farms, Inc. (Germantown, NY) for use in the 2-year studies. Rats and mice were quarantined for 11 days before the beginning of the studies. Six male and six female rats and three male and four female mice were selected for parasite evaluation and gross observation of disease. Serology samples were selected for viral screening. Rats were approximately 45 days old and mice were approximately 40 days old at the beginning of the studies. The health of the animals was monitored during the studies according to the protocols of the NTP Sentinel Animal Program (Appendix I).

Animal Maintenance

Rats and mice were housed individually. Feed and water were available *ad libitum*. Cages and racks were rotated every 2 weeks. Further details of animal maintenance are provided in Table 1. Information on feed composition and contaminants is provided in Appendix H.

Clinical Examinations and Pathology

All animals were observed twice daily for moribundity and mortality. Clinical findings were recorded monthly and body weights were recorded weekly through week 10, once during week 12, and monthly thereafter.

A complete necropsy was performed on all rats and mice. At the 15-month interim evaluation, the left kidney, right kidney, and liver of rats and mice were weighed. At necropsy, all organs and tissues were examined for grossly visible lesions, and all major tissues were fixed and preserved in 10% neutral buffered formalin, processed and trimmed, embedded in paraffin, sectioned to a thickness of 5 to 6 μm , and stained with hematoxylin and eosin for microscopic examination. Complete histopathologic examinations were performed on skin from the site of application and control skin in all animals. Otherwise, complete histopathologic examinations were performed only on control and 1.5 mg/kg animals. Tissues examined microscopically are listed in Table 1.

Microscopic evaluations were completed by the study laboratory pathologist, and the pathology data were entered into the Toxicology Data Management System. The microscope slides, paraffin blocks, and residual wet tissues were sent to the NTP Archives for inventory, slide/block match, and wet tissue audit. The slides, individual animal data records, and pathology tables were evaluated by an independent quality assessment laboratory. The individual animal records and tables were compared for accuracy, the slide and tissue counts were verified, and the histotechnique was evaluated. For the 2-year studies, a quality assessment pathologist reviewed sections of treated and untreated skin.

The quality assessment report and slides were submitted to the NTP Pathology Working Group (PWG) chair, who reviewed the selected tissues and any other tissues for which a disagreement in diagnosis between the laboratory and quality assessment pathologists existed. Representative histopathology slides containing examples of skin lesions related to chemical administration, examples of disagreements in diagnoses between the laboratory and quality assessment pathologist, or lesions of general interest were presented by the chair to the PWG for review. The PWG consisted of the quality assessment pathologist and other pathologists experienced in rodent toxicologic pathology. This group examined the tissues without any knowledge of dose groups or previously rendered diagnoses. When the PWG consensus differed from the opinion of the laboratory pathologist, the diagnosis was changed. Thus, the final diagnoses represent a consensus of contractor pathologists and the PWG. Details of these review procedures have been described, in part, by Maronpot and Boorman (1982) and Boorman *et al.* (1985). For subsequent analyses of the pathology data, the diagnosed lesions for each tissue type were evaluated separately or combined according to the guidelines of McConnell *et al.* (1986).

Statistical Methods

Survival Analyses

The probability of survival was estimated by the product-limit procedure of Kaplan and Meier (1958) and is presented in the form of graphs. Animals found dead of other than natural causes or missexed were censored from the survival analyses; animals dying from natural causes were not censored. Statistical

analyses for possible dose-related effects on survival used Cox's (1972) method for testing two groups for equality and Tarone's (1975) life table test to identify dose-related trends. All reported P values for the survival analyses are two sided.

Calculation of Incidence

The incidences of neoplasms or nonneoplastic lesions as presented in Tables A1, A4, B1, B4, C1, C4, D1, and D4 are given as the number of animals bearing such lesions at a specific anatomic site and the number of animals with that site examined microscopically. For calculation of statistical significance, the incidences of most neoplasms (Tables A3, B3, C3, and D3) and all nonneoplastic lesions are given as the ratio of the number of affected animals to the number of animals with the site examined microscopically. However, when macroscopic examination was required to detect neoplasms in certain tissues (e.g., skin, intestine, harderian gland, and mammary gland) before microscopic evaluation, or when neoplasms had multiple potential sites of occurrence (e.g., leukemia or lymphoma), the denominators consist of the number of animals on which a necropsy was performed.

Analysis of Neoplasm Incidences

The majority of neoplasms in these studies were considered to be incidental to the cause of death or not rapidly lethal. Thus, the primary statistical method used was logistic regression analysis, which assumed that the diagnosed neoplasms were discovered as the result of death from an unrelated cause and thus did not affect the risk of death. In this approach, neoplasm prevalence was modeled as a logistic function of chemical exposure and time. Both linear and quadratic terms in time were incorporated initially, and the quadratic term was eliminated if the fit of the model was not significantly enhanced. The neoplasm incidences of exposed and control groups were compared on the basis of the likelihood score test for the regression coefficient of dose. This method of adjusting for intercurrent mortality is the prevalence analysis of Dinse and Lagakos (1983), further described and illustrated by Dinse and Haseman (1986). When neoplasms are incidental, this comparison of the time-specific neoplasm prevalences also provides a comparison of the time-specific neoplasm incidences (McKnight and Crowley, 1984).

Other methods of statistical analysis were used, and the results of these tests are summarized in the appendixes. These methods include the life table test (Cox, 1972; Tarone, 1975), appropriate for rapidly lethal neoplasms, and the Fisher exact test and the Cochran-Armitage trend test (Armitage, 1971; Gart *et al.*, 1979), procedures based on the overall proportion of neoplasm-bearing animals.

Tests of significance included pairwise comparisons of each dosed group with controls and a test for an overall dose-related trend. Continuity-corrected tests were used in the analysis of neoplasm incidence, and reported P values are one sided. The procedures described in the preceding paragraphs were also used to evaluate selected nonneoplastic lesions. For further discussion of these statistical methods, refer to Haseman (1984).

Analysis of Nonneoplastic Lesion Incidences

Because all nonneoplastic lesions in this study were considered to be incidental to the cause of death or not rapidly lethal, the primary statistical analysis used was a logistic regression analysis in which nonneoplastic lesion prevalence was modeled as a logistic function of chemical exposure and time. For lesions detected at the interim evaluation, the Fisher exact test was used, a procedure based on the overall proportion of affected animals.

Analysis of Continuous Variables

Organ and body weight data, which have approximately normal distributions, were analyzed using the parametric multiple comparison procedures of Dunnett (1955) and Williams (1971, 1972). Jonckheere's test (Jonckheere, 1954) was used to assess the significance of the dose-related trends and to determine whether a trend-sensitive test (Williams' or Shirley's test) was more appropriate for pairwise comparisons than a test that does not assume a monotonic dose-related trend (Dunnett's or Dunn's test). Average severity values were analyzed for significance using the Mann-Whitney U test (Hollander and Wolfe, 1973).

Quality Assurance Methods

The 13-week and 2-year studies were conducted in compliance with Food and Drug Administration Good Laboratory Practice Regulations (21 CFR, Part 58). In addition, as records from the 2-year studies were submitted to the NTP Archives, these studies were audited retrospectively by an independent quality assurance contractor. Separate audits covering completeness and accuracy of the pathology data, pathology specimens, final pathology tables, and a draft of this NTP Technical Report were conducted. Audit procedures and findings are presented in the reports and are on file at NIEHS. The audit findings were reviewed and assessed by NTP staff, so all comments had been resolved or were otherwise addressed during the preparation of this Technical Report.

GENETIC TOXICOLOGY

The genetic toxicity of benzethonium chloride was assessed by testing the ability of the chemical to induce mutations in *Salmonella typhimurium*, and sister chromatid exchanges and chromosomal aberrations in Chinese hamster ovary cells. The protocols and results for these studies are given in Appendix E.

The genetic toxicity studies of benzethonium chloride are part of a larger effort by the NTP to develop a database that would permit the evaluation of carcinogenicity in experimental animals from the structure and responses of the chemical in short-term *in vitro* and *in vivo* genetic toxicity tests. These genetic toxicity tests were originally developed to study mechanisms of chemically induced DNA damage and to predict carcinogenicity in animals, based on the electrophilic theory of chemical carcinogenesis and the somatic mutation theory (Miller and Miller, 1977; Straus, 1981; Crawford, 1985).

There is a strong correlation between a chemical's potential electrophilicity (structural alert to DNA reactivity), mutagenicity in *Salmonella*, and carcinogenicity in rodents. The combination of electrophilicity and *Salmonella* mutagenicity is highly correlated with the induction of carcinogenicity in rats and mice and/or at multiple tissue sites (Ashby and Tennant, 1991). Other *in vitro* genetic toxicity tests do not

correlate well with rodent carcinogenicity (Tennant *et al.*, 1987; Zeiger *et al.*, 1990), although these other tests can provide information on the types of DNA and chromosome effects that can be induced by the chemical being investigated. Data from NTP studies show that a positive response in *Salmonella* is currently the most predictive *in vitro* test for rodent carcinogenicity (89% of the *Salmonella* mutagens were rodent carcinogens), and that there is no complementarity among the *in vitro* genetic toxicity tests. That is, no battery of tests that included the *Salmonella* test improved the predictivity of the *Salmonella* test alone. The predictivity for carcinogenicity of a positive response in bone marrow chromosome aberration or micronucleus tests is not yet defined.

TABLE 1
Experimental Design and Materials and Methods in the Dermal Studies of Benzethonium Chloride

16-Day Studies	13-Week Studies	2-Year Studies
Study Laboratory Battelle Columbus Laboratories (Columbus, OH)	Battelle Columbus Laboratories (Columbus, OH)	Battelle Columbus Laboratories (Columbus, OH)
Strain and Species Rats: F344/N Mice: B6C3F ₁	Rats: F344/N Mice: B6C3F ₁	Rats: F344/N Mice: B6C3F ₁
Animal Source Simonsen Laboratories, Inc. (Gilroy, CA)	Simonsen Laboratories, Inc. (Gilroy, CA)	Taconic Farms, Inc. (Germantown, NY)
Time Held Before Studies Rats: 11 days Mice: 12 days	Rats: 28 days Mice: 27 days	11 days
Average Age When Studies Began Rats: 36-42 days Mice: 37-43 days	Rats: 53-60 days Mice: 58 days	Rats: 45 days Mice: 40 days
Date of First Dose Rats: 17 December 1984 Mice: 18 December 1984	Rats: 2 or 3 May 1985 Mice: 1 May 1985	Rats: 15 June 1987 Mice: 22 June 1987
Duration of Dosing 16 days (5 days/week, excluding holidays)	91 days (5 days/week, excluding holidays)	104 weeks (5 days/week, excluding holidays)
Date of Last Dose Rats: 1 January 1985 Mice: 2 January 1985	Rats: 31 July or 1 August 1985 Mice: 29 or 30 July 1985	Rats: 2 June 1989 Mice: 9 June 1989
Necropsy Dates Rats: 2 January 1985 Mice: 3 January 1985	Rats: 1 or 2 August 1985 Mice: 30 or 31 July 1985	Rats: 12-14 June 1989 Mice: 19-23 June 1989
Average Age at Necropsy Rats: 53-59 days Mice: 54-60 days	Rats: 144-152 days Mice: 149 days	Rats: 111 weeks Mice: 110 weeks
Size of Study Groups 5 males and 5 females	10 males and 10 females	60 males and 60 females
Method of Distribution Animals randomized from weight classes using a computer-generated list of random numbers	Same as 16-day studies	Same as 16-day studies
Animals per Cage 1	1	1

TABLE 1
Experimental Design and Materials and Methods in the Dermal Studies of Benzethonium Chloride
 (continued)

16-Day Studies	13-Week Studies	2-Year Studies
Method of Animal Identification		
Toe clip	Rats: toe clip and cage card Mice: toe mark	Toe clip
Diet		
NIH-07 open formula pellet diet (Zeigler Brothers, Inc., Gardners, PA), available <i>ad libitum</i>	Same as 16-day studies	Same as 16-day studies
Water Distribution		
Tap water (Columbus municipal supply) via automatic watering system (Edstrom Industries, Waterford, WI), available <i>ad libitum</i>	Same as 16-day studies	Same as 16-day studies
Cages		
Polycarbonate (Lab Products, Inc., Garfield, NJ), changed once weekly	Same as 16-day studies	Same as 16-day studies
Bedding		
BetaChip heat-treated hardwood chips (Northeastern Products, Inc., Warrensburg, NY), changed once weekly	BetaChip heat-treated hardwood chips (Northeastern Products, Inc., Warrensburg, NY), changed twice weekly	BetaChip heat-treated hardwood chips (Northeastern Products, Inc., Warrensburg, NY) through the week of 22 May 1988; SaniChip hardwood bedding (P.J. Murphy Forest Products, Corp., Montville, NJ) from the week of 22 May 1988 until the end of the study; changed once weekly
Cage Filters		
Spun-bonded polyester (DuPont 2024) (Snow Filtration, Cincinnati, OH), changed once every 2 weeks	Same as 16-day studies	Same as 16-day studies
Racks		
Stainless steel (Lab Products, Inc., Garfield, NJ), changed once every 2 weeks	Same as 16-day studies	Same as 16-day studies
Animal Room Environment		
Temperature: 21°-24° C Relative humidity: 35%-65% Fluorescent light: 12 hours/day Room air: 15 changes/hour	Temperature: 21°-24° C Relative humidity: 35%-65% Fluorescent light: 12 hours/day Room air: 15 changes/hour	Temperature: 21°-24° C Relative humidity: 35%-65% Fluorescent light: 12 hours/day Room air: 10 changes/hour

TABLE 1
Experimental Design and Materials and Methods in the Dermal Studies of Benzethonium Chloride
 (continued)

16-Day Studies	13-Week Studies	2-Year Studies
Doses Rats: 0, 6.3, 12.5, 25, 50, or 100 mg/kg in 250 μ L ethanol Mice: 0, 6.3, 12.5, 25, 50, or 100 mg/kg in 100 μ L ethanol	Rats: 0, 1.563, 3.125, 6.25, 12.5, or 25 mg/kg in not more than 300 μ L of ethanol Mice: 0, 1.563, 3.125, 6.25, 12.5, or 25 mg/kg in not more than 100 μ L of ethanol	Rats: 0, 0.15, 0.5, or 1.5 mg/kg in not more than 296 μ L (males) or 317 μ L (females) of ethanol Mice: 0, 0.15, 0.5, or 1.5 mg/kg in not more than 131 μ L of ethanol
Type and Frequency of Observation Observed twice daily; animals were weighed initially, on day 10, and at the end of the studies; clinical observations recorded on dosing days	Observed twice daily; body weights and clinical observations recorded weekly	Observed twice daily; body weights recorded weekly through week 10, once during week 12, and monthly thereafter; clinical observations recorded monthly
Method of Sacrifice CO ₂ asphyxiation	CO ₂ asphyxiation	CO ₂ asphyxiation
Necropsy A necropsy was performed on all animals. Organs weighed were brain, heart, right kidney, liver, lung, right testis (rats), and thymus.	A necropsy was performed on all animals. Organs weighed were brain, heart, right kidney, liver, lungs, right testis, and thymus.	A necropsy was performed on all animals. Organs weighed at the 15-month interim evaluation were left kidney, right kidney, and liver.
Histopathology Histopathology was performed on all rats and mice. In addition to gross lesions and tissue masses, skin samples from the site of application and undosed skin samples were examined	Complete histopathology was performed on all control and 25 mg/kg rats and mice. In addition to gross lesions and tissue masses, the tissues examined included: adrenal gland, brain, clitoral or preputial gland, esophagus, femur, including marrow, gallbladder (mice), heart, large intestine (cecum, colon, rectum), small intestine (duodenum, jejunum, and ileum), kidney, liver, lung, mammary gland, mandibular or mesenteric lymph node, nose, ovary, pancreas, parathyroid gland, pituitary gland, prostate gland, salivary gland, spinal cord and sciatic nerve, spleen, stomach, testis with epididymis and seminal vesicle, thymus, thyroid gland, trachea, urinary bladder, and uterus. In addition, skin samples (from the site of application and from unexposed sites) from all dose groups were examined microscopically	Complete histopathology was performed on all control and 1.5 mg/kg rats and mice. In addition to gross lesions and tissue masses, the tissues examined included: adrenal gland, brain, clitoral or preputial gland, esophagus, femur, including marrow, gallbladder (mice), heart, large intestine (cecum, colon, rectum), small intestine (duodenum, jejunum, and ileum), kidney, liver, lung, mammary gland, mandibular or mesenteric lymph node, nose, ovary, pancreas, parathyroid gland, pituitary gland, prostate gland, salivary gland, spinal cord and sciatic nerve, spleen, stomach, testis with epididymis and seminal vesicle, thymus, thyroid gland, trachea, urinary bladder, and uterus. In addition, skin samples (from the site of application and from unexposed sites) from all dose groups were examined microscopically

RESULTS

RATS

16-DAY STUDY

All rats survived to the end of the study (Table 2). The final mean body weights and weight gains of males and females administered 50 or 100 mg benzethonium chloride/kg body weight were significantly less than those of the controls. At necropsy, thickening or hardening of the skin at the site of application was observed in all rats administered 50 or 100 mg/kg and in 25 mg/kg males. Lesions at the site of application appeared crusty or red-grey in color.

Organ weights appeared generally appropriate for body weight in the various dose groups of males and females with the exception of thymus weights, both relative and absolute, which were decreased in 100 mg/kg males and females and in 50 mg/kg females (Table F1).

Histopathologic lesions of the skin varied from minimal epithelial hyperplasia to severe necrotizing, ulcerative lesions penetrating the epidermis to involve the underlying dermis and subcutaneous tissues. The proliferative lesions of the skin at the site of application are collectively referred to as epithelial hyperplasia. Epithelial hyperplasia consisted of a spectrum of epidermal proliferative lesions (in order of greater to lesser frequency): acanthosis, hyperkeratosis, basal cell hyperplasia, and/or increase in the stratum granulosum. Often concomitant with epithelial hyperplasia, and in particular with increasing dose, was evidence of: parakeratosis, sebaceous gland hyperplasia, chronic inflammation, necrosis, and/or ulceration. Sebaceous gland hyperplasia is often considered a component of epithelial hyperplasia but is described separately (along with inflammation, necrosis, and/or ulceration) for interpretive purposes. Sebaceous gland hyperplasia was defined to include both an apparent increase in either the number of cells within a gland or an increase in the number of glands observed per unit area.

Ulcerative, necrotizing inflammation of the epidermis of marked severity was a consistent feature in 50 and 100 mg/kg males and females. Chronic inflammation of mild to moderate severity in the dermis and subcutaneous tissues was also generally present in these animals. Similar lesions of lesser severity were also observed in 25 mg/kg male and female rats. In 6.3 and 12.5 mg/kg males and females, the predominant lesion consisted of minimal to mild epithelial hyperplasia (primarily hyperkeratosis) with minimal evidence of inflammation of the epidermis and/or dermis. Epithelial hyperplasia was observed in three males and two females administered 6.3 mg/kg and in four males and two females administered 12.5 mg/kg.

Dose Selection Rationale: The severity of the skin lesions and the lower body weights observed in 50 and 100 mg/kg males and females precluded the use of doses greater than 25 mg/kg. Adaptation of the skin may occur following chronic topical administration of an apparent irritant, and thus a high dose of 25 mg/kg was selected for the 13-week study in male and female rats.

TABLE 2
Survival and Body Weights of Rats in the 16-Day Dermal Study of Benzethonium Chloride

Dose (mg/kg)	Survival ^a	Mean Body Weight ^b (g)			Final Weight Relative to Controls (%)
		Initial	Final	Change	
Male					
0	5/5	97 ± 4	167 ± 7	70 ± 4	
6.3	5/5	96 ± 3	165 ± 5	69 ± 2	98
12.5	5/5	95 ± 3	162 ± 7	67 ± 4	97
25	5/5	94 ± 4	154 ± 4	59 ± 3*	92
50	5/5	93 ± 4	140 ± 6**	48 ± 3**	84
100	5/5	94 ± 4	127 ± 6**	33 ± 3**	76
Female					
0	5/5	86 ± 25	123 ± 3	37 ± 30	
6.3	5/5	85 ± 39	120 ± 3	35 ± 10	98
12.5	5/5	86 ± 37	124 ± 2	37 ± 16	101
25	5/5	83 ± 34	116 ± 3	33 ± 27	94
50	5/5	84 ± 37	109 ± 5*	26 ± 35**	89
100	5/5	83 ± 37	108 ± 6*	25 ± 33**	88

* Significantly different ($P \leq 0.05$) from the control group by Williams' or Dunnett's test.

** $P \leq 0.01$

^a Number of animals surviving/number initially in group

^b Weights and weight changes are given as mean ± standard error.

13-WEEK STUDY

All rats survived to the end of the study (Table 3). The final mean body weights and weight gain of 25 mg/kg males were significantly lower than those of the controls. The final mean body weights of all other groups of males and of all groups of females were similar to those of the controls. Clinical findings included crusting, apparent thickening, and reddening of the skin (irritation) at the site of application in groups administered 3.125 mg/kg or greater.

The absolute and relative thymus weights of 25 mg/kg males were significantly less than those of the controls (Table F2); the absolute and relative right kidney weights of 25 mg/kg females were significantly greater than those of the controls.

In groups of rats receiving 6.25 mg/kg or greater doses, histopathologic lesions of the skin varied from epithelial hyperplasia and inflammation of mild to moderate severity to necrotizing, ulcerative lesions penetrating the epidermis to involve the underlying dermis and subcutaneous tissues (Table 4). Similar lesions of lesser (minimal) severity were observed in 3.125 mg/kg males and females. In 1.563 mg/kg male and female rats, minimal epithelial hyperplasia and chronic inflammation of the epidermis were observed in up to half of the animals.

In the bone marrow, hypercellularity of the myeloid fraction was noted in 25 mg/kg male and female rats. This observation was considered a secondary response, indicative of the extent and duration of the inflammation at 25 mg/kg, and was not evaluated at lower doses.

TABLE 3
Survival and Body Weights of Rats in the 13-Week Dermal Study of Benzethonium Chloride

Dose (mg/kg)	Survival ^a	Mean Body Weight ^b (g)			Final Weight Relative to Controls (%)
		Initial	Final	Change	
Male					
0	10/10	177 ± 6	320 ± 6	143 ± 3	
1.563	10/10	178 ± 6	319 ± 6	141 ± 5	100
3.125	10/10	180 ± 5	327 ± 6	147 ± 4	102
6.25	10/10	176 ± 5	315 ± 6	139 ± 5	98
12.5	10/10	177 ± 5	312 ± 4	135 ± 4	97
25	10/10	176 ± 5	288 ± 6**	112 ± 3**	90
Female					
0	10/10	119 ± 2	180 ± 3	61 ± 3	
1.563	10/10	121 ± 2	181 ± 3	59 ± 2	100
3.125	10/10	119 ± 2	176 ± 2	57 ± 1	98
6.25	10/10	121 ± 2	177 ± 3	56 ± 2	98
12.5	10/10	120 ± 2	177 ± 3	57 ± 2	98
25	10/10	120 ± 2	176 ± 3	56 ± 2	98

** Significantly different ($P \leq 0.01$) from the control group by Williams' or Dunnett's test.

^a Number of animals surviving/number initially in group

^b Weights and weight changes are given as mean ± standard error.

TABLE 4
Incidences of Nonneoplastic Lesions of the Skin in Rats in the 13-Week Dermal Study
of Benzethonium Chloride

Dose	Vehicle Control	1.563 mg/kg	3.125 mg/kg	6.25 mg/kg	12.5 mg/kg	25 mg/kg
Male						
Skin (Site of Application) ^a	10	10	10	10	10	10
Epithelial hyperplasia ^b	0	4* (1.0) ^c	9** (1.1)	10** (1.7)	10** (2.6)	10** (3.0)
Inflammation, chronic	0	2 (1.0)	7** (1.1)	9** (1.7)	10** (2.5)	10** (3.5)
Necrosis	0	0	1 (1.0)	2 (1.0)	6** (1.2)	9** (1.9)
Ulceration	0	0	2 (1.0)	4* (1.5)	8** (1.9)	10** (2.5)
Female						
Skin (Site of Application)	10	10	10	9	10	10
Epithelial hyperplasia	0	5* (1.0)	9** (1.4)	9** (1.6)	10** (1.9)	10** (3.0)
Inflammation, chronic	0	4* (1.0)	10** (1.5)	7** (1.6)	10** (1.7)	10** (3.2)
Necrosis	0	0	1 (1.0)	3 (1.3)	5* (1.0)	8** (1.4)
Ulceration	0	0	5* (1.8)	3 (1.7)	1 (1.0)	10** (2.0)

* Significantly different ($P \leq 0.05$) from the control group by the Fisher exact test.

** ($P \leq 0.01$)

^a Number of animals with skin examined microscopically.

^b Number of animals with lesion.

^c Average severity grade of lesions in affected animals (1=minimal; 2=mild; 3=moderate; 4=marked)

Dose Selection Rationale: The frequency of epithelial hyperplasia, chronic inflammation, necrosis, and/or ulcerations in males and females administered 3.125 mg/kg or greater, combined with the apparent progression of skin lesions (rather than adaptation) between the 16-day and 13-week studies (more animals and lower dose groups were affected), suggested 3.125 mg/kg body weight may be excessive for a chronic study. Thus, a high dose of 1.5 mg/kg was selected for the 2-year study conducted in male and female rats.

2-YEAR STUDY

Survival

Estimates of 2-year survival probabilities for male and female rats are shown in Table 5 and in the Kaplan-Meier survival curves (Figure 1). Survival rates of all dosed groups of males and females were similar to those of the controls.

Body Weights and Clinical Findings

Mean body weights of all dosed groups of males and females were similar to those of the controls throughout the study (Tables 6 and 7, Figure 2), and the final mean body weights of all dosed groups of males and females were also similar to those of the controls. There were no significant differences in liver or kidney weights in males or females evaluated at 15 months (Table F3). Reddening of the skin was observed at the site of application in all dosed groups of males and females; crusts were observed in 0.5 mg/kg males and in 1.5 mg/kg females.

TABLE 5
Survival of Rats in the 2-Year Dermal Study of Benzethonium Chloride

	Vehicle Control	0.15 mg/kg	0.5 mg/kg	1.5 mg/kg
Male				
Animals initially in study	60	60	60	60
15-Month interim evaluation ^a	8	8	5	4
Accidental death ^a	0	0	1	0
Moribund	25	34	34	25
Natural deaths	12	7	11	15
Animals surviving to study termination	15	11	9 ^c	16
Percent probability of survival at end of study ^b	29	21	17	29
Mean survival (days) ^c	627	605	599	615
Survival analysis ^d	P=0.892N	P=0.286	P=0.129	P=0.802
Female				
Animals initially in study	60	60	60	60
15-Month interim evaluation ^a	9	7	9	7
Moribund	13	11	13	13
Natural deaths	14	9	12	16
Animals surviving to study termination	24	33	26	24
Percent probability of survival at end of study	47	63	51	46
Mean survival days	638	652	641	630
Survival analysis	P=0.256	P=0.232N	P=0.854N	P=0.728

^a Censored from survival analyses

^b Kaplan-Meier determinations based on the number of animals alive on the first day of terminal sacrifice

^c Mean of all deaths (uncensored, censored, and terminal sacrifice)

^d The result of the life table trend test (Tarone, 1975) is in the control column, and the results of the life table pairwise comparisons (Cox, 1972) with the controls are in the dosed columns. A negative trend or a lower mortality in a dose group is indicated by N.

^e Includes one animal that died during the last week of the study

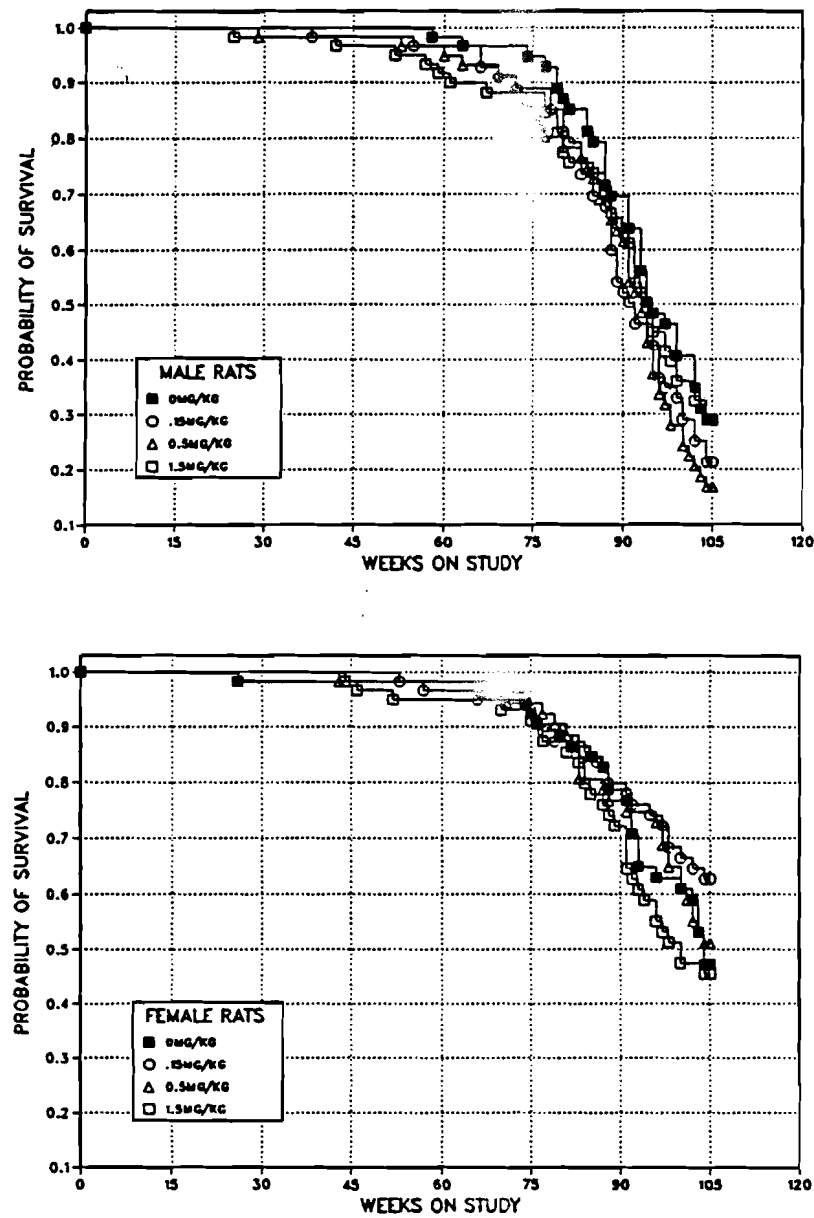


FIGURE 1
Kaplan-Meier Survival Curves for Male and Female Rats
Administered Benzethonium Chloride Topically for 2 Years

TABLE 6
Mean Body Weights and Survival of Male Rats in the 2-Year Dermal Study of Benzethonium Chloride

Weeks on Study	Vehicle Control		0.15 mg/kg			0.5 mg/kg			1.5 mg/kg		
	Av. WL (g)	No. of Survivors	Av. WL (g)	WL (% of controls)	No. of Survivors	Av. WL (g)	WL (% of controls)	No. of Survivors	Av. WL (g)	WL (% of controls)	No. of Survivors
1	131	60	132	101	60	129	98	60	130	100	60
2	165	60	166	100	60	164	99	60	165	100	60
3	197	60	197	100	60	196	99	60	197	100	60
4	225	60	225	100	60	223	99	60	225	100	60
5	245	60	246	100	60	244	100	59	247	101	60
6	261	60	265	101	60	262	100	59	264	101	60
7	278	60	279	100	60	277	100	59	277	100	60
8	294	60	295	100	60	294	100	59	293	100	60
9	309	60	310	100	60	308	100	59	307	99	60
10	318	60	320	101	60	318	100	59	318	100	60
12	336	60	336	100	60	333	99	59	334	99	60
16	364	60	363	100	60	362	99	59	357	98	60
20	386	60	387	100	60	384	99	59	377	98	60
24	398	60	402	101	60	398	100	59	389	98	60
28	415	60	419	101	60	413	100	59	401	97	59
32	431	60	432	100	60	428	99	58	414	96	59
36	443	60	443	100	60	440	99	58	424	96	59
40	449	60	451	100	59	448	100	58	434	97	59
44	456	60	458	100	59	455	100	58	440	96	58
48	466	60	467	100	59	466	100	58	450	96	58
52	479	60	475	99	59	475	99	58	458	96	58
56	491	60	487	99	58	486	99	57	471	96	57
60	495	59	492	99	58	487	98	57	476	96	55
64	497	58	493	99	58	490	99	55	480	97	54
68 ^a	490	50	488	100	48	482	98	50	473	96	49
72	493	50	491	100	47	476	97	49	475	96	49
76	494	49	485	98	46	478	97	44	467	95	49
80	478	46	468	98	44	465	98	43	460	96	45
84	467	44	466	100	38	454	97	41	453	97	42
88	458	37	451	99	35	439	96	37	441	96	39
92	452	33	452	100	25	439	97	29	431	95	34
96	439	25	425	97	22	416	95	20	427	97	25
100	410	21	415	101	17	414	101	15	416	102	20
104	396	16	396	100	13	396	100	10	385	97	18
Mean for weeks											
1-13	251		252	100		250	100		251	100	
14-52	429		430	100		427	100		414	97	
53-104	466		462	99		456	98		450	97	

^a Interim evaluation occurred during week 66.

TABLE 7
Mean Body Weights and Survival of Female Rats in the 2-Year Dermal Study of Benzethonium Chloride

Weeks on Study	Vehicle Control		0.15 mg/kg			0.5 mg/kg			1.5 mg/kg		
	Av. WL (g)	No. of Survivors	Av. WL (g)	WL (% of controls)	No. of Survivors	Av. WL (g)	WL (% of controls)	No. of Survivors	Av. WL (g)	WL (% of controls)	No. of Survivors
1	110	60	108	98	60	109	99	60	108	98	60
2	127	60	125	98	60	126	99	60	127	100	60
3	141	60	139	98	60	140	99	60	141	100	60
4	152	60	152	100	60	151	99	60	153	100	60
5	162	60	161	100	60	160	99	60	162	100	60
6	170	60	167	99	60	167	98	60	169	100	60
7	175	60	173	99	60	171	98	60	174	100	60
8	183	60	181	99	60	178	97	60	182	100	60
9	188	60	185	98	60	184	98	60	187	99	60
10	191	60	189	99	60	187	98	60	190	99	60
12	197	60	195	99	60	194	99	60	196	100	60
16	206	60	204	99	60	202	98	60	204	99	60
20	212	60	213	100	60	209	98	60	211	99	60
24	217	60	216	99	60	213	98	60	214	99	60
28	228	59	228	100	60	223	98	60	223	98	60
32	236	59	236	100	60	230	98	60	230	98	60
36	241	59	241	100	60	233	97	60	233	97	60
40	247	59	249	101	60	240	97	60	240	97	60
44	251	59	253	101	60	246	98	59	245	98	59
48	262	59	264	101	60	256	98	59	254	97	58
52	273	59	272	100	60	265	97	59	263	96	57
56	280	59	280	100	59	273	97	59	268	96	57
60	287	59	289	101	58	281	98	59	276	96	57
64	291	59	292	101	58	283	97	59	280	96	57
68 ^a	294	50	296	101	50	287	98	49	280	95	50
72	296	49	301	102	50	291	98	48	280	95	49
76	299	46	305	102	49	293	98	48	284	95	48
80	302	45	308	102	46	293	97	46	285	94	46
84	300	44	311	104	45	296	99	41	286	95	44
88	301	41	312	104	44	300	100	39	288	96	40
92	302	39	316	105	40	302	100	38	287	95	34
96	309	33	316	103	39	295	96	38	290	94	30
100	306	32	317	104	36	295	96	32	291	95	27
104	299	26	322	108	34	295	99	27	289	97	25
Mean for weeks											
1-13	163		161	99		161	99		163	100	
14-52	237		238	100		232	98		232	98	
53-104	297		305	103		291	98		283	95	

^a Interim evaluation occurred during week 66.

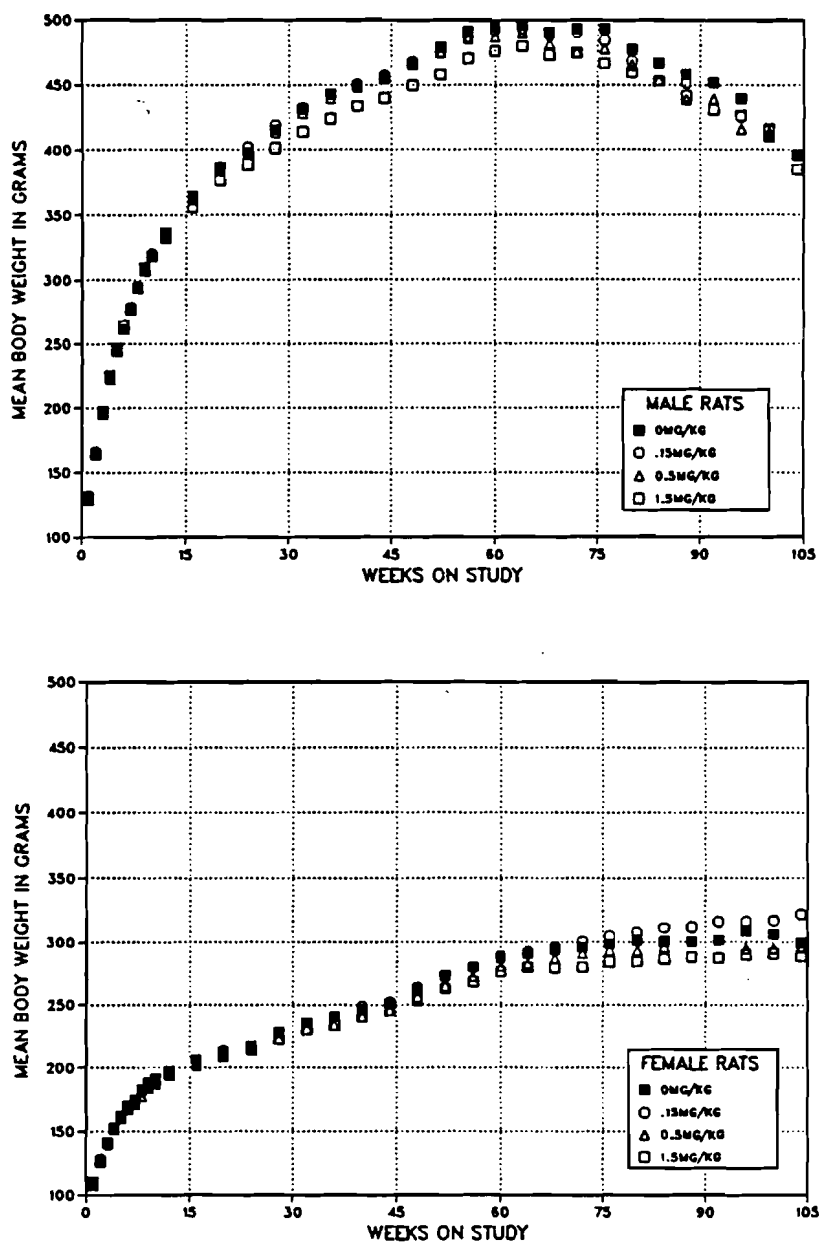


FIGURE 2
Growth Curves for Male and Female Rats Administered Benzethonium Chloride Topically for 2 Years

Pathology and Statistical Evaluation

This section describes the statistically significant or biologically noteworthy changes in the incidences of neoplasms and nonneoplastic lesions in the skin. Summaries of the incidences of neoplasms and nonneoplastic lesions, individual animal tumor diagnoses, and statistical analyses of primary neoplasms that occurred with an incidence of at least 5% in at least one animal group are presented in Appendix A for male rats and Appendix B for female rats.

No neoplasms considered to be chemical-related were observed in male or female rats. In particular, no skin-associated neoplasms were attributed to treatment with benzethonium chloride. Two incidences of keratoacanthoma and one of sebaceous gland carcinoma were observed in treated males (Table 8). These neoplasms were consistent with the spectrum of neoplasms found in adjacent control skin of treated and untreated animals (Table A1). No neoplasms were found in females at the site of application (Table B1).

Treatment-related nonneoplastic lesions were observed at the site of application and varied from minimal evidence of epithelial hyperplasia with or without evidence of sebaceous gland hyperplasia, to more advanced cases of mild to moderate epithelial hyperplasia accompanied by evidence of ulceration. Sebaceous gland hyperplasia was commonly observed in 1.5 mg/kg females and to a lesser extent in 0.5 mg/kg females at both the 15-month interim evaluation and the end of the 2-year study. With rare exception, sebaceous gland hyperplasia was associated with cases of epithelial hyperplasia and generally was a coexistent finding with the more severely affected cases of epithelial hyperplasia involving inflammation and/or ulceration. Microscopic evidence of epidermal ulceration was observed in one 1.5 mg/kg male and four 1.5 mg/kg females at the 15-month interim evaluation. While this lesion was not observed in males at the end of the study, epidermal ulceration was noted in females, particularly at 1.5 mg/kg (Table 8).

TABLE 8
Incidences of Skin Neoplasms and Nonneoplastic Lesions in Rats in the 2-Year Dermal Study of Benzethonium Chloride

Dose	Vehicle Control	0.15 mg/kg	0.5 mg/kg	1.5 mg/kg
Male				
15-Month Interim Evaluation				
Skin (Site of Application) ^a	8	8	5	4
Epithelial hyperplasia ^{b,c}	0	0	3* (1.3) ^d	4** (2.3)
Sebaceous gland, hyperplasia	0	0	2 (2.0)	2 (1.5)
Ulceration	0	0	0	1 (1.0)
2-Year Study				
Skin (Site of Application)	52	52	55	56
Epithelial hyperplasia	1 (1.0)	0	4 (1.0)	10** (1.6)
Sebaceous gland, hyperplasia	0	0	2 (1.5)	2 (1.5)
Ulceration	0	0	0	0
Keratoacanthoma ^e	0	0	1	1
Sebaceous gland, carcinoma	0	0	1	0
Female				
15-Month Interim Evaluation				
Skin (Site of Application)	9	7	9	7
Epithelial hyperplasia	0	1 (3.0)	2 (2.0)	6** (2.7)
Sebaceous gland, hyperplasia	0	1 (2.0)	1 (2.0)	6** (2.0)
Ulceration	0	1 (2.0)	1 (2.0)	4* (2.3)
2-Year Study				
Skin (Site of Application)	51	53	51	53
Epithelial hyperplasia	2 (2.0)	2 (3.5)	6 (3.0)	32** (2.7)
Sebaceous gland, hyperplasia	1 (1.0)	2 (2.5)	6 (1.7)	30** (1.9)
Ulceration	0	1 (3.0)	3 (3.3)	19** (1.8)

* Significantly different ($P \leq 0.05$) from the control group by Fisher's exact test (15-month interim evaluation) or logistic regression test (2-year study).

** ($P \leq 0.01$)

^a Number of animals with skin examined microscopically.

^b Number of animals with lesion.

^c Epithelial hyperplasia was originally diagnosed as acanthosis and/or hyperkeratosis.

^d Average severity grade of lesions in affected animals (1=minimal; 2=mild; 3=moderate; 4=marked)

^e All neoplasms found at the site of application are included for completeness. The incidences of neoplasms were not statistically significant, nor were any neoplasms considered to be related to chemical treatment.

MICE

16-DAY STUDY

One 100 mg/kg male mouse died on day 4 of the study; all other mice survived to the end of the study (Table 9). The mean body weight gains of males administered 25, 50, or 100 mg benzethonium chloride/kg body weight were significantly greater than that of the control; final mean body weights of all groups of dosed males and females were similar to those of the controls. Mild crusting, scaling and reddening of the skin at the site of application was observed in 50 and 100 mg/kg male and female groups and in 25 mg/kg males.

The absolute heart weights of 100 mg/kg males and females and the relative heart weight of 100 mg/kg females were significantly greater than those of the controls (Table F4). The absolute and relative thymus weights of 100 mg/kg females were significantly decreased.

Necrotizing inflammation of the epidermis (moderate to marked in males, and mild to moderate in females), with inflammatory involvement of the underlying dermis and subcutaneous tissues was a consistent feature in 100 mg/kg male and female mice. Although of lesser severity (minimal to mild), this spectrum of lesions of the dermis and epidermis was also observed in three 50 mg/kg males and two 50 mg/kg females. The predominant lesion in 12.5 and 25 mg/kg males and females and in 6.3 mg/kg females consisted of minimal to mild epithelial hyperplasia with minimal evidence of inflammatory involvement of the epidermis extending into the dermis in some animals. Minimal epithelial hyperplasia without evidence of inflammation was observed in 6.25 mg/kg male mice. Histopathologic lesions of the skin were not observed in vehicle control males (ethanol); however, minimal epithelial hyperplasia was noted in two control females.

TABLE 9
Survival and Body Weights of Mice in the 16-Day Dermal Study of Benzethonium Chloride

Dose (mg/kg)	Survival ^a	Mean Body Weight ^b (g)			Final Weight Relative to Controls (%)
		Initial	Final	Change	
Male					
0	5/5	23.9 ± 0.7	25.1 ± 0.4	1.2 ± 0.7	
6.3	5/5	24.1 ± 0.6	25.3 ± 0.6	1.2 ± 0.1	101
12.5	5/5	23.9 ± 0.5	25.9 ± 0.4	1.9 ± 0.2	103
25	5/5	23.4 ± 0.7	26.5 ± 0.5	3.0 ± 0.4*	105
50	5/5	23.9 ± 0.7	26.4 ± 0.7	2.5 ± 0.2*	105
100	4/5 ^c	23.7 ± 0.7	26.5 ± 0.5	2.4 ± 0.2*	105
Female					
0	5/5	18.0 ± 0.3	21.2 ± 0.5	3.2 ± 0.2	
6.3	5/5	18.0 ± 0.2	21.6 ± 0.4	3.5 ± 0.3	102
12.5	5/5	18.0 ± 0.3	21.0 ± 0.4	3.1 ± 0.4	99
25	5/5	18.1 ± 0.6	21.3 ± 0.3	3.2 ± 0.4	101
50	5/5	17.8 ± 0.3	21.1 ± 0.3	3.3 ± 0.0	99
100	5/5	17.8 ± 0.3	20.9 ± 0.5	3.1 ± 0.3	99

* Significantly different ($P \leq 0.05$) from the control group by Williams' or Dunnett's test.

^a Number of animals surviving/number initially in group

^b Weights and weight changes are given as mean ± standard error.

^c Day of death: 4

Dose Selection Rationale: Because of the severity of the skin lesions in mice administered 50 mg/kg or greater, 25 mg/kg was selected as the high dose for the 13-week study in male and female mice.

13-WEEK STUDY

All mice survived to the end of the study (Table 10). The final mean body weights of all dosed groups of males and females were similar to those of the controls; the mean weight gain of 25 mg/kg males was significantly less than that of the controls. Males administered 6.25, 12.5, or 25 mg benzethonium chloride/kg body weight developed crusting, thickening of the skin, scales, and reddening of the skin at the site of application, as did female mice administered 12.5 or 25 mg/kg.

The absolute and relative right kidney and liver weights of 12.5 and 25 mg/kg males were slightly greater than those of the controls (Table F5).

Histopathologic lesions of the skin at the site of application varied from a minimal epithelial hyperplasia with chronic inflammation, to mild epithelial hyperplasia with chronic inflammation of the epidermis and focal necrosis of the epithelium and involvement of the underlying dermis and subcutaneous tissues (Table 11). Chronic inflammation and necrosis of the epidermis of minimal to mild severity was observed in 25 mg/kg males. Necrosis was a feature of the spectrum of lesions observed in one 6.25 mg/kg male and in two 25 mg/kg females. All dosed groups of male and female mice exhibited evidence of chemical treatment; epithelial hyperplasia (with or without inflammation) was observed in nine 1.563 mg/kg males and females.

TABLE 10
Survival and Body Weights of Mice in the 13-Week Dermal Study of Benzethonium Chloride

Dose (mg/kg)	Survival ^a	Mean Body Weight ^b (g)			Final Weight Relative to Controls (%)
		Initial	Final	Change	
Male					
0	10/10	23.9 ± 0.6	32.7 ± 1.1	8.9 ± 0.8	
1.563	10/10	23.8 ± 0.5	31.9 ± 0.8	8.1 ± 0.7	97
3.125	10/10	23.7 ± 0.5	32.2 ± 1.0	8.5 ± 0.6	98
6.25	10/10	23.8 ± 0.3	31.8 ± 0.5	7.9 ± 0.5	97
12.5	10/10	23.6 ± 0.4	31.3 ± 0.9	7.7 ± 0.5	96
25	10/10	24.0 ± 0.5	30.6 ± 0.7	6.6 ± 0.5*	93
Female					
0	10/10	18.8 ± 0.3	26.2 ± 0.8	7.4 ± 0.7	
1.563	10/10	18.7 ± 0.2	26.4 ± 0.6	7.7 ± 0.5	101
3.125	10/10	19.4 ± 0.4	27.0 ± 0.6	7.6 ± 0.4	103
6.25	10/10	18.9 ± 0.2	26.8 ± 0.6	7.9 ± 0.6	102
12.5	10/10	19.4 ± 0.4	25.8 ± 0.6	6.4 ± 0.4	98
25	10/10	19.4 ± 0.5	26.4 ± 0.9	7.0 ± 0.5	101

* Significantly different ($P \leq 0.05$) from the control group by Williams' or Dunnett's test.

^a Number of animals surviving/number initially in group

^b Weights and weight changes are given as mean ± standard error.

TABLE 11
Incidences of Nonneoplastic Lesions of the Skin in Mice in the 13-Week Dermal Study
of Benzethonium Chloride

Dose	Vehicle Control	1.563 mg/kg	3.125 mg/kg	6.25 mg/kg	12.5 mg/kg	25 mg/kg
Male						
Skin (Site of Application) ^a	10	10	10	10	10	10
Epithelial hyperplasia ^b	0	9** (1.0) ^c	8** (1.0)	9** (1.1)	9** (1.6)	10** (2.2)
Inflammation, chronic	0	2 (1.0)	3 (1.0)	6** (1.0)	9** (1.2)	10** (2.0)
Necrosis	0	0	0	1 (1.0)	1 (1.0)	5* (1.6)
Female						
Skin (Site of Application)	10	10	10	10	10	10
Epithelial hyperplasia	0	9** (1.0)	10** (1.0)	10** (1.0)	10** (1.5)	10** (2.0)
Inflammation, chronic	1 (1.0)	6* (1.0)	8** (1.0)	8** (1.1)	10** (1.6)	10** (1.9)
Necrosis	0	0	0	0	0	2 (1.0)

* Significantly different ($P \leq 0.05$) from the control group by the Fisher exact test.

** ($P \leq 0.01$)

^a Number of animals with skin examined microscopically.

^b Number of animals with lesion.

^c Average severity grade of lesions in affected animals (1=minimal; 2=mild; 3=moderate; 4=marked)

Dose Selection Rationale: Although the skin lesions observed in mice were not as severe as those observed in the 13-week rat study, there was concern over the frequency of inflammatory involvement in male and particularly female mice at the lowest dose tested, 1.563 mg/kg. Inflammation at the site of application, combined with some evidence of progression of the lesions between the 16-day and 13-week studies with lower dose groups exhibiting necrosis and inflammation, led to selection of a high dose of 1.5 mg/kg body weight for the 2-year study.

2-YEAR STUDY

Survival

Estimates of 2-year survival probabilities for male and female mice are shown in Table 12 and in the Kaplan-Meier survival curves (Figure 3). Survival rates of all dosed groups of males and females were similar to those of the controls.

Body Weights and Clinical Findings

Mean body weights of all dosed groups of males and females were similar to those of the controls throughout the study (Tables 13 and 14, Figure 4). Final mean body weights of all dosed male and female groups were also similar to those of the controls. There were no marked changes in liver or kidney weights of dosed mice at the 15-month interim evaluation (Table F6). Reddening of the skin was observed at the site of application in all dosed groups of males and in 0.15 mg/kg females; crusts were observed in 0.5 mg/kg females.

TABLE 12
Survival of Mice in the 2-Year Dermal Study of Benzethonium Chloride

	Vehicle Control	0.15 mg/kg	0.5 mg/kg	1.5 mg/kg
Male				
Animals initially in study	60	60	60	60
15-Month interim evaluation ^a	10	9	9	10
Missexed ^a	0	1	1	0
Moribund	3	8	4	9
Natural deaths	4	4	4	2
Animals surviving to study termination	43	38	42	39
Percent probability of survival at end of study ^b	86	76	84	78
Mean survival (days) ^c	674	675	678	672
Survival analysis ^d	P=0.671	P=0.320	P=0.990	P=0.449
Female				
Animals initially in study	60	60	60	60
15-Month interim evaluation ^a	8	7	10	6
Accidental death ^a	1	0	0	0
Missexed ^a	0	0	2	0
Moribund	10	4	8	13
Natural deaths	3	15	9	7
Animals surviving to study termination	38	34	31	34
Percent probability of survival at end of study	75	65	65	64
Mean survival days	669	640	660	646
Survival analysis	P=0.406	P=0.222	P=0.373	P=0.226

^a Censored from survival analyses

^b Kaplan-Meier determinations based on the number of animals alive on the first day of terminal sacrifice

^c Mean of all deaths (uncensored, censored, and terminal sacrifice)

^d The result of the life table trend test (Tarone, 1975) is in the control column, and the results of the life table pairwise comparisons (Cox, 1972) with the controls are in the dosed columns.

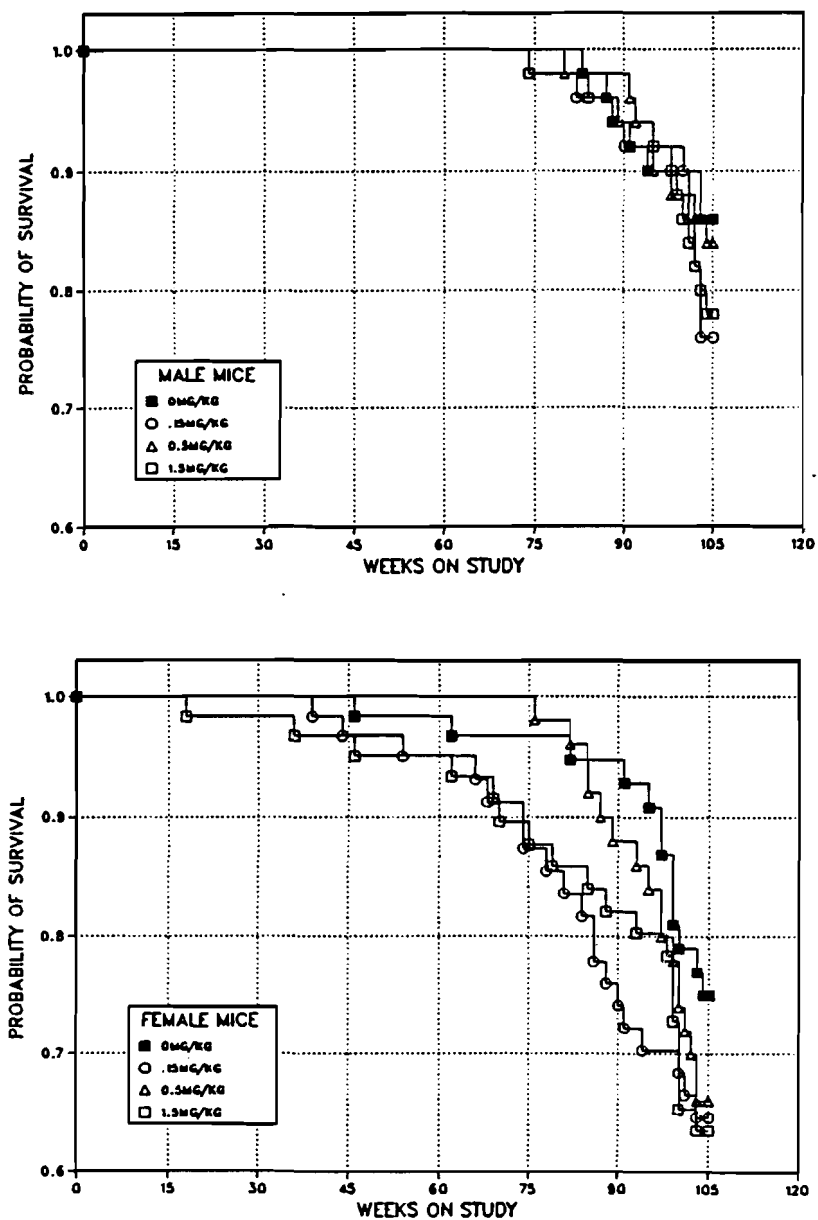


FIGURE 3
Kaplan-Meier Survival Curves for Male and Female Mice
Administered Benzethonium Chloride Topically for 2 Years

TABLE 13
Mean Body Weights and Survival of Male Mice in the 2-Year Dermal Study of Benzethonium Chloride

Weeks on Study	Vehicle Control		0.15 mg/kg			0.5 mg/kg			1.5 mg/kg		
	Av. WL (g)	No. of Survivors	Av. WL (g)	WL (% of controls)	No. of Survivors	Av. WL (g)	WL (% of controls)	No. of Survivors	Av. WL (g)	WL (% of controls)	No. of Survivors
1	23.1	60	22.8	99	60	23.5	101	60	23.2	100	60
2	24.7	60	24.1	98	60	24.5	99	60	24.4	99	60
3	25.5	60	24.8	97	60	25.7	100	60	25.5	100	60
4	26.3	60	25.6	97	60	25.9	99	60	25.9	99	60
5	27.2	60	26.4	97	60	26.9	99	60	26.9	99	60
6	28.4	60	27.9	98	60	28.0	100	60	28.6	101	60
7	29.2	60	28.4	97	60	28.7	99	60	28.9	99	60
8	29.6	60	29.1	98	60	29.5	100	60	29.7	100	60
9	30.3	60	29.7	98	60	30.3	100	60	30.4	100	60
10	31.1	60	30.5	98	60	31.1	100	60	30.9	99	60
12	32.4	60	31.6	98	60	32.5	100	60	32.6	101	60
16	34.4	60	33.6	98	60	34.0	99	60	34.4	100	60
20	36.6	60	35.5	97	60	35.7	98	60	36.7	100	60
24	38.9	60	37.3	96	60	38.2	98	60	38.6	99	60
28	40.8	60	39.5	97	60	40.9	100	60	41.2	101	60
32	42.5	60	40.9	96	60	42.5	100	60	42.5	100	60
36	44.7	60	43.3	97	60	45.0	101	60	45.0	101	60
40	46.0	60	44.6	97	60	45.5	99	60	46.0	100	60
44	46.4	60	45.4	98	60	46.1	99	60	46.7	101	60
48	46.9	60	46.5	99	60	46.7	100	60	47.5	101	60
52	48.4	60	47.6	98	60	48.2	100	60	48.9	101	60
56	48.4	60	47.8	99	60	48.3	100	60	49.2	102	60
60	49.4	60	48.7	99	60	49.2	100	60	50.0	101	60
64	49.8	60	49.0	98	60	49.6	100	60	50.3	101	60
68 ^a	49.5	50	49.0	99	51	49.8	101	51	50.3	102	50
72	50.0	50	49.1	98	51	50.9	102	51	51.2	102	50
76	50.3	50	49.9	99	50	51.0	101	51	51.7	103	49
80	51.1	50	50.9	100	50	51.4	101	51	52.5	103	49
84	51.5	49	50.9	99	49	51.5	100	50	51.6	100	49
88	51.2	48	50.1	98	49	50.3	98	50	50.4	98	48
92	51.0	46	49.7	98	47	49.8	98	48	49.7	98	47
96	51.4	45	50.0	97	47	51.2	100	46	49.5	96	46
100	49.8	45	47.6	96	47	50.5	101	45	48.8	98	43
104	48.3	43	49.2	102	39	49.6	103	44	48.5	100	40
Mean for weeks											
1-13	28.0		27.4	98		27.9	100		27.9	100	
14-52	42.6		41.4	97		42.3	99		42.8	100	
53-104	50.1		49.4	99		50.2	100		50.3	100	

^a Interim evaluation occurred during week 66.

TABLE 14
Mean Body Weights and Survival of Female Mice in the 2-Year Dermal Study of Benzethonium Chloride

Weeks on Study	Vehicle Control		0.15 mg/kg			0.5 mg/kg			1.5 mg/kg		
	Av. WL (g)	No. of Survivors	Av. WL (g)	WL (% of controls)	No. of Survivors	Av. WL (g)	WL (% of controls)	No. of Survivors	Av. WL (g)	WL (% of controls)	No. of Survivors
1	18.9	60	18.5	98	60	18.5	98	60	18.5	98	60
2	20.2	60	19.9	99	60	19.8	98	60	19.9	99	60
3	21.2	60	21.4	101	60	21.0	99	60	20.7	98	60
4	21.9	60	21.8	100	60	22.0	101	60	22.3	102	60
5	22.8	60	23.0	101	60	22.7	100	60	22.8	100	60
6	24.1	60	24.4	101	60	24.1	100	60	24.2	100	60
7	24.8	60	24.9	100	60	24.9	100	60	24.8	100	60
8	25.4	60	25.9	102	60	25.9	102	60	25.6	101	60
9	26.5	60	26.4	100	60	26.4	100	60	26.4	100	60
10	27.2	60	27.3	100	60	27.3	100	60	27.3	100	60
12	28.3	60	28.7	101	60	28.9	102	60	28.7	101	60
16	29.7	60	29.4	99	60	30.5	103	60	30.0	101	60
20	31.7	60	31.0	98	60	32.1	101	60	31.5	99	59
24	34.0	60	34.0	100	60	34.4	101	60	33.3	98	59
28	36.9	60	36.2	98	60	36.9	100	60	36.7	100	59
32	38.1	60	37.5	98	60	38.2	100	60	37.4	98	59
36	40.6	60	39.7	98	60	40.9	101	60	40.1	99	59
40	42.3	60	41.7	99	59	42.6	101	60	41.7	99	58
44	43.4	60	42.3	98	59	42.9	99	60	42.1	97	58
48	45.1	59	43.8	97	58	44.4	98	60	43.7	97	57
52	47.2	59	45.4	96	58	46.0	98	60	45.1	96	57
56	48.1	59	46.7	97	57	47.0	98	60	46.0	96	57
60	49.4	59	48.1	97	57	48.3	98	60	47.4	96	57
64	50.3	58	48.8	97	57	49.0	97	60	48.5	96	56
68 ^a	50.5	50	48.4	96	48	49.0	97	50	48.6	96	50
72	51.7	50	49.4	96	48	50.2	97	50	50.5	96	48
76	52.7	50	50.8	96	46	51.5	98	49	50.0	95	47
80	54.1	50	51.8	96	45	52.6	97	49	52.6	97	46
84	53.5	49	50.9	95	44	51.8	97	48	52.0	97	46
88	52.8	49	50.1	95	41	51.7	98	45	51.3	97	45
92	50.1	47	49.2	98	38	50.1	100	44	49.4	99	44
96	49.5	46	49.1	99	37	50.7	102	42	48.8	99	43
100	47.2	41	47.0	100	37	48.2	102	39	47.2	100	38
104	46.6	38	46.1	99	34	48.7	105	33	46.9	101	34
Mean for weeks											
1-13	23.8		23.8	100		23.8	100		23.7	100	
14-52	38.9		38.1	98		38.9	100		38.2	98	
53-104	50.5		49.0	97		49.9	99		49.9	99	

^a Interim evaluation occurred during week 66.

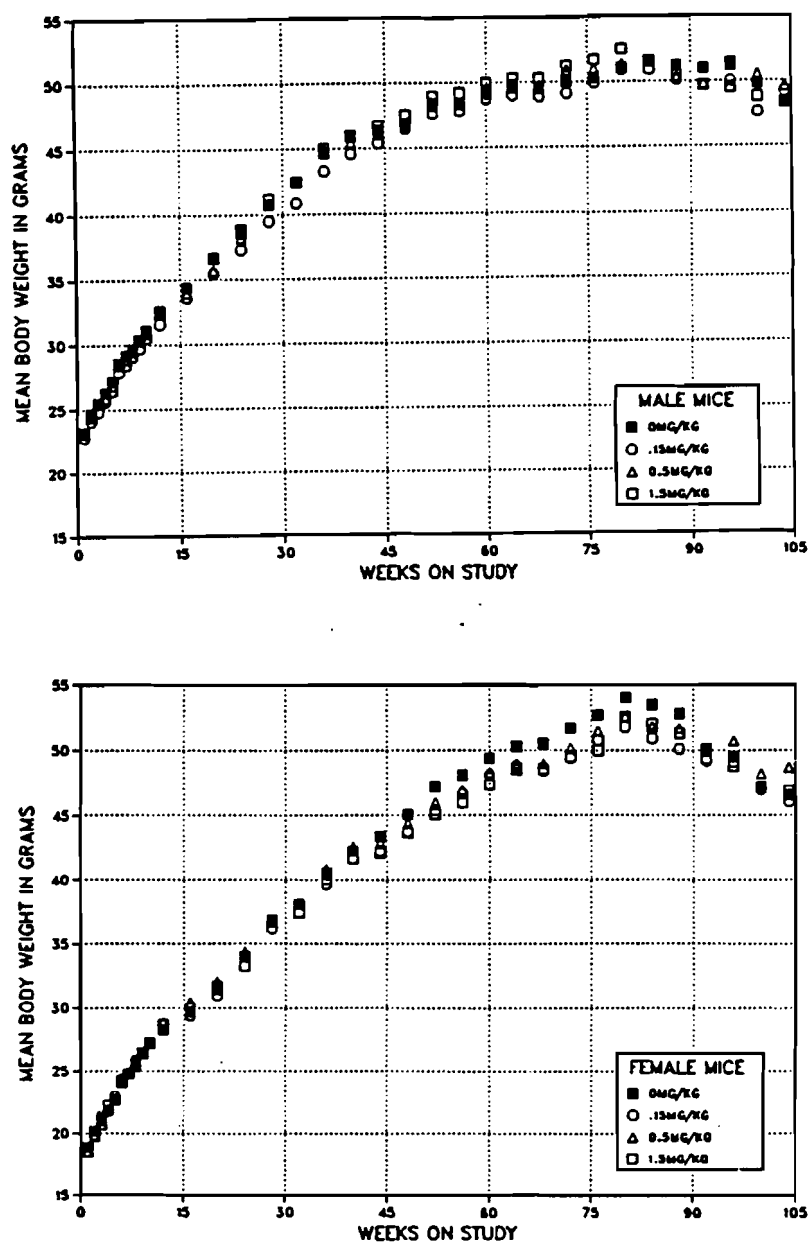


FIGURE 4
Growth Curves for Male and Female Mice Administered Benzethonium Chloride Topically for 2 Years

Pathology and Statistical Evaluation

This section describes the statistically significant or biologically noteworthy changes in the incidences of neoplasms and nonneoplastic lesions in the skin. Summaries of the incidences of neoplasms and nonneoplastic lesions, individual animal tumor diagnoses, and statistical analyses of primary neoplasms that occurred with an incidence of at least 5% in at least one animal group are presented in Appendix C for male mice and Appendix D for female mice.

No neoplasms were observed which were considered related to chemical treatment, including no significant increases in incidences of neoplasms at the site of application. Two subcutaneous neoplasms were found in control females at the site of application: a hemangioma and a sarcoma. In addition, one subcutaneous sarcoma was found in one 1.5 mg/kg female (Tables 15 and D1). No neoplasms were found in male mice at the site application (Table C1), and the spectrum of neoplasms found in control skin of treated male and female mice was consistent with similar neoplasms found in vehicle controls.

The predominant treatment-related histopathologic nonneoplastic lesion observed at the site of application was epithelial hyperplasia of minimal to mild severity (Tables 15, C4, and D4). Epithelial hyperplasia was commonly observed in 1.5 mg/kg male and female mice at the 15-month interim evaluation. Similarly at the end of the study, a dose-response increase in the incidence of epithelial hyperplasia was observed in male and female mice. Unlike the female rat, the progression of the skin lesion with development of ulceration was not observed in male or female mice in this study.

TABLE 15
Incidences of Skin Neoplasms and Nonneoplastic Lesions in Mice in the 2-Year Dermal Study of Benzethonium Chloride

Dose	Vehicle Control	0.15 mg/kg	0.5 mg/kg	1.5 mg/kg
Male				
15-Month Interim Evaluation				
Skin (Site of Application) ^a	10	9	9	10
Epithelial hyperplasia ^{b,c}	0	0	2 (1.0) ^d	10** (1.2)
2-Year Study				
Skin (Site of Application)	50	50	50	50
Epithelial hyperplasia	2 (1.5)	7 (1.1)	16** (1.6)	23** (1.2)
Inflammation, chronic	0	0	0	2 (1.5)
Sebaceous gland, hyperplasia	0	0	1 (1.0)	0
Ulceration	1 (2.0)	1 (2.0)	4 (2.5)	2 (2.0)
Female				
15-Month Interim Evaluation				
Skin (Site of Application)	8	7	10	6
Epithelial hyperplasia	0	0	3 (1.0)	4* (1.3)
2-Year Study				
Skin (Site of Application)	52	52	48	53
Epithelial hyperplasia	3 (1.3)	7 (1.3)	6 (2.2)	22** (1.3)
Inflammation, chronic	1 (1.0)	2 (1.0)	0	0
Sebaceous gland, hyperplasia	0	0	1 (3.0)	0
Ulceration	0	0	2 (3.0)	0
Subcutaneous tissue, hemangioma ^e	1	0	0	0
Subcutaneous tissue, sarcoma	1	0	0	1

* Significantly different ($P \leq 0.05$) from the control group by Fisher's exact test (15-month interim evaluation) or logistic regression test (2-year study).

** ($P \leq 0.01$)

^a Number of animals with skin examined microscopically.

^b Number of animals with lesion.

^c Epithelial hyperplasia was originally diagnosed as acanthosis and/or hyperkeratosis.

^d Average severity grade of lesions in affected animals (1=minimal; 2=mild; 3=moderate; 4=marked)

^e All neoplasms found at the site of application are included for completeness. The incidences of neoplasms were not statistically significant, nor were any neoplasms considered to be related to chemical treatment.

GENETIC TOXICOLOGY

Benzethonium chloride (0.01 to 100 $\mu\text{g}/\text{plate}$) was not mutagenic in *Salmonella typhimurium* strains TA98, TA100, TA1535, or TA1537 (Table E1). All tests were performed with a preincubation protocol, with and without Aroclor 1254-induced male Sprague Dawley rat or Syrian hamster liver S9. In cytogenetic tests with cultured Chinese hamster ovary cells, benzethonium chloride did not induce sister chromatid exchanges (Table E2) or chromosomal aberrations (Table E3), with or without S9. Although increases in chromosomal aberrations were observed in each of the two trials conducted, the increases were not statistically significant or dose related. No cell cycle delay was observed in either cytogenetic test.

DISCUSSION AND CONCLUSIONS

The toxicity and carcinogenicity studies described in this report were performed because benzethonium chloride is found in a variety of over-the-counter products resulting in widespread human exposure, and because of a report of possible carcinogenicity of benzethonium chloride in mice receiving subcutaneous injections of the chemical (Mason *et al.*, 1971). Quaternary ammonium compounds such as benzethonium chloride have antimicrobial properties (CIR, 1985), and some have cholinergic agonist or antagonist actions (Hume and Holland, 1965; Strycker and Long, 1969).

Although these studies were performed using dermal exposure, the results of absorption and disposition studies (NTP, 1988) suggested uptake of at least half of 0.15 to 1.5 mg/kg ^{14}C -labeled doses of benzethonium chloride from the skin, when applied under a non-occlusive patch. Thus, unless significant chemical breakdown occurs on the skin, systemic effects of a toxic or pharmacologic nature would be expected to be demonstrated in these studies.

In the 16-day studies, there were no clinical signs suggestive of systemic cholinergic effects; however, one male mouse receiving 100 mg/kg died after 4 days. There is no reported LD_{50} in mice for dermally applied benzethonium chloride. Homberger (1968) administered as much as 280 mg/kg in a single application to unshaved backs of C57BL/6 male mice without lethal effects. The intravenous LD_{50} for mice is reported in the 20 to 35 mg/kg range (CIR, 1985), so it would appear possible that repeated dermal application of 100 mg/kg could account for the one observed death. Body weight gains of treated rats were decreased at the higher doses, and this was probably related to the rather severe skin lesions produced at these dose levels.

There were no deaths or evidence of systemic toxicity in the 13-week studies. The high doses used in these studies were one-quarter those used in the 16-day studies, and body weight gains were only decreased in high-dose male rats and mice. Again, this was likely secondary to the skin lesions produced by exposure to

benzethonium chloride during the studies. Hypercellularity of the bone marrow was diagnosed in 25 mg/kg rats. This was considered a secondary manifestation of the inflammation occurring at the site of application.

There was little histopathology attributed to exposure to benzethonium chloride in the short- or long-term studies in rats or mice other than a spectrum of skin lesions at the site of application. These lesions were characteristic of lesions observed with a variety of skin irritants, and included components of epithelial hyperplasia and inflammation. The lesions were in general both dose-, and to a lesser extent, time-dependent. However, some skin healing and/or adaptation did occur as illustrated by the fact that a larger percentage of the treated animals had epithelial hyperplasia at the end of the 13-week study than at the end of the 2-year study. In contrast to the results of the 2-year studies in male rats and male and female mice, skin lesions in treated female rats appeared to progress in severity during the 2-year study, with significant evidence of ulceration present in treated females from this study. This is an atypical finding in that the response of rats (NTP, 1994a) and mice (NTP, 1993, 1994a,b) to skin irritants has been very similar in males and females in other NTP studies.

There was no evidence of treatment-related increased incidences of neoplasms at any site in rats or mice. Skin neoplasms occurring at the site of application included keratoacanthomas in one 0.5 mg/kg male rat and in one 1.5 mg/kg male rat, and one sebaceous gland carcinoma in one 0.5 mg/kg male rat. There was no evidence of an increased incidence of skin neoplasms in the treated groups when combining skin neoplasms that occurred at the site of application with those occurring away from the site of application. The results of genetic toxicity studies also indicated that benzethonium chloride is not mutagenic or otherwise genotoxic, thus there would appear to be little concern that repeated exposure to benzethonium chloride at these concentrations is carcinogenic.

As indicated previously, benzethonium chloride at doses of 3.125 mg/kg and above in the rat caused dermatotoxicity in the 13-week study, as evidenced by epithelial hyperplasia, inflammatory infiltration, and ulceration of the epidermis. At higher doses, this ulceration and inflammation became more severe and

involved the deeper dermal and subcutaneous tissues. The irritant property of benzethonium chloride has been observed previously in studies in which dilute solutions of the chemical were administered twice weekly into the subcutaneous tissue of rats (Mason *et al.*, 1971). At doses of 1 and 3 mg/kg, a high number of animals had severe granulomatous reactions at the injection site. By 50 weeks, eight of sixty 1 mg/kg and sixteen of eighty 3 mg/kg rats developed locally invasive, non-metastatic sarcomas (primarily fibrosarcomas); none was present in the 50 controls. While clearly a treatment-related effect, it is difficult to evaluate the degree to which the marked inflammatory component may have contributed to the increase in the incidence of neoplasms. Full-thickness damage or "wounding" of the skin of mice has been shown to induce epithelial hyperplasia, inflammation of the dermis and epidermis, and the marked proliferation of granulation tissue. In long-term initiation/promotion studies the "wounding" has been shown to be a highly effective skin neoplasm promotion regimen (Argyris, 1989). 12-*O*-Tetradecanoyl-phorbol-13-acetate (TPA), often considered the positive control in skin neoplasm promotion studies, is known to induce a low incidence of skin neoplasms when administered alone at doses which also induce skin irritation (NTP, 1994b,c). Following initiation with DMBA the most effective doses of TPA for skin neoplasm promotion are also those doses which result in epithelial erosion, inflammation, and regenerative epithelial hyperplasia (Argyris, 1989). Recent skin initiation/promotion studies with TPA have shown that the most sensitive strains of mice to neoplasm promotion were also those that were significantly more sensitive to the irritancy of the chemical, as evidenced by a marked inflammatory reaction (NTP, 1994c). Lower doses of TPA or lesser epidermal damage such as repeated mild abrasion of the skin surface with fine sandpaper, although adequate to induce some epithelial hyperplasia, were not shown to induce a deeper inflammatory component or to be sufficient to promote neoplasms following similar skin neoplasm initiation with DMBA (Argyris, 1989). Although epithelial hyperplasia was observed in male and female rats and mice in the present studies, and ulcerative lesions were observed in female rats, it is unknown whether higher doses capable of inducing more severe, necrotizing inflammatory skin lesions may have resulted in increases in skin-associated neoplasia.

CONCLUSIONS

Under the conditions of these 2-year dermal studies, there was *no evidence of carcinogenic activity** of benzethonium chloride in male or female F344/N rats receiving 0.15, 0.5, or 1.5 mg/kg. There was *no evidence of carcinogenic activity* in male or female B6C3F₁ mice receiving 0.15, 0.5, or 1.5 mg/kg.

Exposure of rats and mice to benzethonium chloride by dermal application in ethanol for 2 years resulted in epithelial hyperplasia in male and female rats and mice and sebaceous gland hyperplasia and ulcers in female rats at the site of application.

* Explanation of Levels of Evidence of Carcinogenic Activity is on page 11.

REFERENCES

- The Aldrich Library of NMR Spectra*. (1983). 2nd ed., Vol. 5. Aldrich Chemical Company, Inc., Milwaukee, WI. Spectra No. 136B.
- American Medical Association (AMA) (1980). *AMA Drug Evaluations*. 3rd ed. p. 890. American Medical Association, Chicago, IL.
- Argyris, T.S. (1989). Epidermal tumor promotion by damage in the skin of mice. In *Skin Carcinogenesis: Mechanisms and Human Relevance* (T.J. Slaga, *et al.*, Eds.), pp. 63-80. Alan R. Liss, Inc., New York.
- Armitage, P. (1971). *Statistical Methods in Medical Research*, pp. 362-365. John Wiley and Sons, New York.
- Ashby, J., and Tennant, R.W. (1991). Definitive relationships among chemical structure, carcinogenicity, and mutagenicity for 301 chemicals tested by the U.S. NTP. *Mutat. Res.* **257**, 229-306.
- Boorman, G.A., Montgomery, C.A., Jr., Eustis, S.L., Wolfe, M.J., McConnell, E.E., and Hardisty, J.F. (1985). Quality assurance in pathology for rodent carcinogenicity studies. In *Handbook of Carcinogen Testing* (H.A. Milman and E.K. Weisburger, Eds.), pp. 345-357. Noyes Publications, Park Ridge, NJ.
- Christensen, P.W. (1963). Evaluation of the antibacterial effect of preservatives, with special preference to phemerol and thiomersal. *Acta Pathol. Microbiol. Scand.* **57**, 104-110.
- Code of Federal Regulations (CFR) **21**, Part 58.
- Compton, F.H., and Beagrie, G.S. (1975). Inhibitory effect of benzethonium and zinc chloride mouthrinses on human dental plaque and gingivitis. *J. Clin. Periodontol.* **2**, 33-43.
- Cosmetic Tioletry and Fragrance Association (CTFA) (1985). Final report on the safety assessment of benzethonium chloride and methylbenzethonium chloride. *J. Am. Coll. Toxicol.* **4** (Suppl. 5), 65-106.
- Cox, D.R. (1972). Regression models and life-tables. *J. R. Stat. Soc.* **B34**, 187-220.
- Crawford, B.D. (1985). Perspectives on the somatic mutation model of carcinogenesis. In *Advances in Modern Environmental Toxicology: Mechanisms and Toxicity of Chemical Carcinogens and Mutagens* (M.A. Mehlman, W.G. Flamm, and R.J. Lorentzen, Eds.), pp. 13-59. Princeton Scientific Publishing Co., Inc., Princeton, NJ.
- De Flora, S., Camoirano, A., Zanicchi, P., and Bennicelli, C. (1984a). Mutagenicity testing with TA97 and TA102 of 30 DNA-damaging compounds, negative with other *Salmonella* strains. *Mutat. Res.* **134**, 159-165.
- De Flora, S., Zanicchi, P., Camoirano, A., Bennicelli, C., and Badolati, G.S. (1984b). Genotoxic activity and potency of 135 compounds in the Ames reversion test and in a bacterial DNA-repair test. *Mutat. Res.* **133**, 161-198.
- Dinse, G.E., and Haseman, J.K. (1986). Logistic regression analysis of incidental-tumor data from animal carcinogenicity experiments. *Fundam. Appl. Toxicol.* **6**, 44-52.
- Dinse, G.E., and Lagakos, S.W. (1983). Regression analysis of tumor prevalence data. *Appl. Statist.* **32**, 236-248.

Dunnnett, C.W. (1955). A multiple comparison procedure for comparing several treatments with a control. *J. Am. Stat. Assoc.* **50**, 1095-1121.

Finnegan, J.K., and Dienna, J.B. (1954). Toxicity of quaternaries. *Soap Sanit. Chem.* **30**, 147-175.

Gaffar, A., Marcussen, H.W., Solis-Gaffar, M.C., and Rustogi, K.N. (1980). Long term anti-plaque gingivitis and calculus effects of benzethonium chloride in beagle dogs. *J. Periodont. Res.* **15**, 107-110.

Galloway, S.M., Armstrong, M.J., Reuben, C., Colman, S., Brown, B., Cannon, C., Bloom, A.D., Nakamura, F., Ahmed, M., Duk, S., Rimpo, J., Margolin, B.H., Resnick, M.A., Anderson, B., and Zeiger, E. (1987). Chromosome aberrations and sister chromatid exchanges in Chinese hamster ovary cells: Evaluations of 108 chemicals. *Environ. Mol. Mutagen.* **10** (Suppl. 10), 1-175.

Gart, J.J., Chu, K.C., and Tarone, R.E. (1979). Statistical issues in interpretation of chronic bioassay tests for carcinogenicity. *J. Natl. Cancer Inst.* **62**, 957-974.

Gilman, M.R., and DeSalva, S.J. (1979). Teratology studies of benzethonium chloride, cetyl pyridinium chloride and chlorhexidine in rats. *Toxicol. Appl. Pharmacol.* **48**, 35 (Abstr.).

Haseman, J.K. (1984). Statistical issues in the design, analysis and interpretation of animal carcinogenicity studies. *Environ. Health Perspect.* **58**, 385-392.

Hollander, M., and Wolfe, D.A. (1973). *Nonparametric Statistical Methods*, pp. 120-123. John Wiley and Sons, New York.

Homburger, F. (1968). Carcinogenicity of several compounds. National Technical Information Service, PB. No. 183-027, 26 pp.

Hume, A.S., and Holland, W.C. (1965). Vasopressor and depressor activity of phenylalkyltrimethylammonium compounds. *Arch. Int. Pharmacodyn.* **154**, 155-160.

Jackson, R.L., and Aprison, M.H. (1966). Mammalian brain acetylcholinesterase. Effects of surface-active agents. *J. Neurochem.* **13** (Suppl. 12), 1367-1371.

Johnson, O.H., Casey, S., Doeltz, M.K., McCaleb, K.E., Miller, A.M., Papa, P.A., Swett, L.B., Valentini, M.A., and Helmes, C.T. (1984). A study of biocides for the selection of candidates for carcinogen bioassay. *J. Environ. Sci. Health A19* (Suppl. 1), 1-25.

Jonckheere, A.R. (1954). A distribution-free *k*-sample test against ordered alternatives. *Biometrika* **41**, 133-145.

Kaplan, E.L., and Meier, P. (1958). Nonparametric estimation from incomplete observations. *J. Am. Stat. Assoc.* **53**, 457-481.

Klein, M., and Stevens, D.A. (1945). *In vitro* and *in vivo* activity of synthetic detergents against influenza A virus. *J. Immunol.* **50**, 265-273.

Kuwahara, K., Hitoshi, T., Hayashi, H., and Satake, K. (1976). Effects of detergents on cultured human cells. *Kyushu Yakugakkai Kaiho* **30**, 31-37.

La Rosa, R.T., Fine, N., and De Salva, S.J. (1978). Rat maternal and fetal absorption of ¹⁴C-benzethonium chloride (¹⁴C-BTC). *Pharmacologist* **20**, 254 (Abstr.).

- McConnell, E.E., Solleveld, H.A., Swenberg, J.A., and Boorman, G.A. (1986). Guidelines for combining neoplasms for evaluation of rodent carcinogenesis studies. *JNCI* **76**, 283-289.
- McKnight, B., and Crowley, J. (1984). Tests for differences in tumor incidence based on animal carcinogenesis experiments. *J. Am. Stat. Assoc.* **79**, 639-648.
- Maronpot, R.R., and Boorman, G.A. (1982). Interpretation of rodent hepatocellular proliferative alterations and hepatocellular tumors in chemical safety assessment. *Toxicol. Pathol.* **10**, 71-80.
- Mason, M.M., Cate, C.C., and Baker, J. (1971). Toxicology and carcinogenesis of various chemicals used in the preparation of vaccines. *Clin. Toxicol.* **4** (Suppl. 2), 185-204.
- The Merck Index*. (1976). 9th ed. (M. Windholz, Ed.). Merck and Company, Rahway, NJ.
- Miller, J.A., and Miller, E.C. (1977). Ultimate chemical carcinogens as reactive mutagenic electrophiles. In *Origins of Human Cancer* (H.H. Hiatt, J.D. Watson, and J.A. Winsten, Eds.), pp. 605-627. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
- Mote, E.M., Schoessler, J.P., and Hill, R.M. (1969). Lens incorporated germicides. *J. Am. Optom. Assoc.* **40**, 291-293.
- National Cancer Institute (NCI) (1976). Guidelines for Carcinogen Bioassay in Small Rodents. Technical Report Series No. 1. NIH Publication No. 76-801. U.S. Department of Health, Education, and Welfare, Public Health Service, National Institutes of Health, Bethesda, MD.
- National Institutes of Health (NIH) (1978). Open Formula Rat and Mouse Ration (NIH-07). Specification NIH-11-1335. U.S. Department of Health, Education, and Welfare, Public Health Service, National Institutes of Health, Bethesda, MD.
- National Toxicology Program (NTP) (1988). The Absorption, Distribution, and Elimination of ¹⁴C-Benzethonium Chloride Following IV Administration or a Single or a 10-Day Repeated Dermal Application in Fischer 344 Rats. National Toxicology Program, Research Triangle Park, NC. 32 pp.
- National Toxicology Program (NTP) (1989). Assessment of Contact Hypersensitivity to Benzethonium Chloride in Female B6C3F₁ Mice. National Toxicology Program, Research Triangle Park, NC. 11 pp.
- National Toxicology Program (NTP) (1993). Toxicology and Carcinogenesis Studies of *p*-Nitrophenol (CAS No. 100-02-7) in Swiss-Webster Mice (Dermal Studies). Technical Report Series No. 417. NIH Publication No. 93-3148. U.S. Department of Health and Human Services, Public Health Service, National Institutes of Health, Research Triangle Park, NC.
- National Toxicology Program (NTP) (1994a). Toxicology and Carcinogenesis Studies of Diethylphthalate (CAS No. 84-66-2) in F344/N Rats and B6C3F₁ Mice (Dermal Studies) with Dermal Initiation/Promotion Study of Diethylphthalate and Dimethylphthalate (CAS No. 131-11-3) in Male Swiss (CD-1®) Mice. Technical Report Series No. 429. NIH Publication No. 94-3356. U.S. Department of Health and Human Services, Public Health Service, National Institutes of Health, Research Triangle Park, NC. (in press).
- National Toxicology Program (NTP) (1994b). One-Year Initiation/Promotion Study of *o*-Benzyl-*p*-Chlorophenol (CAS NO. 120-32-1) in Swiss (CD-1®) Mice (Mouse Skin Study). Technical Report Series No. 444. NIH Publication No. 94-3157. U.S. Department of Health and Human Services, Public Health Service, National Institutes of Health, Research Triangle Park, NC. (in press)

National Toxicology Program (NTP) (1994c). Comparative Initiation/Promotion Skin Paint Study in B6C3F₁ Mice, Swiss (CD-1®) Mice, and SENCAR Mice. Technical Report Series No. 441. NIH Publication No. 94-3357. U.S. Department of Health and Human Services, Public Health Service, National Institutes of Health, Research Triangle Park, NC. (in preparation)

Paniagua, M.E., Piedras, R., Vaillant, H.W., and Gamble, C.J. (1961). Field trial of a contraceptive foam in Puerto Rico. *JAMA* 177, 125-129.

Sadtler Standard Spectra. IR No. 22233; UV No. 19579. Sadtler Research Laboratories, Philadelphia.

Stanford Research Institute (SRI) (1984). Chemical Economics Handbook.

Stedman, R.L., Kravitz, E., and King, J.D. (1957). Studies on cell surface-germicide and enzyme-germicide reactions and their contribution to the lethal effect. *J. Bacteriol.* 73, 655-660.

Straus, D.S. (1981). Somatic mutation, cellular differentiation, and cancer causation. *JNCI* 67, 233-421.

Strycker, S.J., and Long, J.P. (1969). Studies on the muscarinic and antimuscarinic activity of benzyltrimethylammonium bromide. *J. Pharmaceutical Sciences* 58, 671-674.

Swan, K.C. (1944). Reactivity of the ocular tissues to wetting agents. *Am. J. Ophthalmol.* 27, 1118-1122.

Tanzer, J.M., Slee, A.M., Kamay, B., and Scheer, E.R. (1979). *In vitro* evaluation of seven cationic detergents as antiplaque agents. *Antimicrob. Agents Chemother.* 15 (Suppl. 3), 408-414.

Tarone, R.E. (1975). Tests for trend in life table analysis. *Biometrika* 62, 679-682.

Tennant, R.W., Margolin, B.H., Shelby, M.D., Zeiger, E., Haseman, J.K., Spalding, J., Caspary, W., Resnick, M., Stasiewicz, S., Anderson, B., and Minor, R. (1987). Prediction of chemical carcinogenicity in rodents from *in vitro* genetic toxicity assays. *Science* 236, 933-941.

U.S. Food and Drug Administration (FDA) (1980a). Status of Ingredients in the OTC Drug Review. Division of OTC Drug Evaluation (HFD-510), Bureau of Drugs.

U.S. Food and Drug Administration (FDA) (1980b). Establishment of a Monograph and Proposed Rulemaking on Ophthalmic Drug Products for Over-the-Counter Human Use. Fed Reg. 45 (May 6, 1989) p. 30,005-30,006, 30,017.

Volpe, A.R., Kupieczak, L.J., Brant, J.H., King, W.J., Kestenbaum, R.C., and Schlissell, H.J. (1969). Antimicrobial control of bacterial plaque and calculus and the effects of these agents on oral flora. *J. Dent. Res.* 48, 832-841.

Williams, D.A. (1971). A test for differences between treatment means when several dose levels are compared with a zero dose control. *Biometrics* 27, 103-117.

Williams, D.A. (1972). The comparison of several dose levels with a zero dose control. *Biometrics* 28, 519-531.

Zeiger, E., Anderson, B., Haworth, S., Lawlor, T., Mortelmans, K., and Speck, W. (1987). *Salmonella* mutagenicity tests: III. Results from the testing of 255 chemicals. *Environ. Mutagen.* 9 (Suppl. 9), 1-110.

Zeiger, E., Haseman, J.K., Shelby, M.D., Margolin, B.H., and Tennant, R.W. (1990). Evaluation of four *in vitro* genetic toxicity tests for predicting rodent carcinogenicity: Confirmation of earlier results with 41 additional chemicals. *Environ. Mol. Mutagen.* 16 (Suppl. 18), 1-14.

APPENDIX A
SUMMARY OF LESIONS IN MALE RATS
IN THE 2-YEAR DERMAL STUDY
OF BENZETHONIUM CHLORIDE

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TABLE A1

Summary of the Incidence of Neoplasms in Male Rats in the 2-Year Dermal Study of Benzethonium Chloride^a

	Vehicle Control	0.15 mg/kg	0.5 mg/kg	1.5 mg/kg
Disposition Summary				
Animals initially in study	60	60	60	60
<i>15-Month interim evaluation</i>	8	8	5	4
Early deaths				
Accidental death			1	
Moribund	25	34	34	25
Natural deaths	12	7	11	15
Survivors				
Died last week of study			1	
Terminal sacrifice	15	11	8	16
Animals examined microscopically	60	60	60	60
15-Month Interim Evaluation				
Alimentary System				
Stomach, forestomach	(1)			
Squamous cell papilloma	1 (100%)			
Endocrine System				
Pituitary gland	(8)			(4)
Pars distalis, adenoma	3 (38%)			2 (50%)
Thyroid gland	(8)			(4)
C-cell, adenoma				2 (50%)
Genital System				
Testes	(8)			(4)
Bilateral, interstitial cell, adenoma	1 (13%)			
Interstitial cell, adenoma	2 (25%)			
Integumentary System				
Skin, control	(8)	(8)	(5)	(4)
Skin, site of application	(8)	(8)	(5)	(4)
Systems Examined With No Neoplasms Observed				
Cardiovascular System				
General Body System				
Hematopoietic System				
Musculoskeletal System				
Nervous System				
Respiratory System				
Special Senses System				
Urinary System				

TABLE A1

Summary of the Incidence of Neoplasms in Male Rats in the 2-Year Dermal Study of Benzethonium Chloride (continued)

	Vehicle Control	0.15 mg/kg	0.5 mg/kg	1.5 mg/kg
2-Year Study				
Alimentary System				
Intestine large, colon				(1)
Adenoma				1 (100%)
Intestine large, cecum	(1)			
Intestine small, duodenum				(3)
Intestine small, jejunum	(1)			(1)
Adenocarcinoma				1 (100%)
Adenoma	1 (100%)			
Liver	(52)			(56)
Histiocytic sarcoma	1 (2%)			
Mesentery	(8)			(8)
Lipoma	1 (13%)			
Fat, histiocytic sarcoma	1 (13%)			
Pancreas	(52)			(56)
Pharynx	(1)			
Palate, squamous cell papilloma	1 (100%)			
Salivary glands	(52)			(56)
Sarcoma	1 (2%)			
Tooth	(2)			
Gingiva, squamous cell carcinoma	1 (50%)			
Cardiovascular System				
Heart	(51)			(56)
Endocrine System				
Adrenal cortex	(28)			(24)
Adenoma	1 (4%)			
Squamous cell carcinoma, metastatic, lung				1 (4%)
Adrenal medulla	(27)			(29)
Pheochromocytoma malignant				1 (3%)
Pheochromocytoma benign	3 (11%)			4 (14%)
Bilateral, pheochromocytoma benign	2 (7%)			3 (10%)
Islets, pancreatic	(52)			(56)
Adenoma	1 (2%)			3 (5%)
Parathyroid gland	(50)			(54)
Adenoma	1 (2%)			
Pituitary gland	(52)			(56)
Pars distalis, adenoma	31 (60%)			23 (41%)
Pars intermedia, adenoma	1 (2%)			
Thyroid gland	(51)			(56)
C-cell, adenoma	6 (12%)			7 (13%)
C-cell, adenoma, multiple	1 (2%)			
Follicular cell, adenoma	1 (2%)			2 (4%)
General Body System				
None				

TABLE A1

Summary of the Incidence of Neoplasms in Male Rats in the 2-Year Dermal Study of Benzethonium Chloride (continued)

	Vehicle Control	0.15 mg/kg	0.5 mg/kg	1.5 mg/kg
2-Year Study (continued)				
Genital System				
Epididymis	(52)			(56)
Preputial gland	(52)			(56)
Adenoma				1 (2%)
Carcinoma	1 (2%)			
Bilateral, carcinoma	1 (2%)			
Prostate	(52)			(56)
Seminal vesicle	(52)			(56)
Testes	(52)			(56)
Bilateral, interstitial cell, adenoma	23 (44%)			30 (54%)
Interstitial cell, adenoma	19 (37%)			10 (18%)
Hematopoietic System				
Bone marrow	(52)			(56)
Femoral, histiocytic sarcoma	1 (2%)			
Lymph node	(7)			(14)
Mediastinal, histiocytic sarcoma	1 (14%)			
Renal, histiocytic sarcoma	1 (14%)			
Thoracic, squamous cell carcinoma, metastatic, lung				1 (7%)
Lymph node, mandibular	(13)			(17)
Hemangiosarcoma	1 (8%)			
Lymph node, mesenteric	(8)			(14)
Spleen	(52)			(56)
Hemangiosarcoma	1 (2%)			
Histiocytic sarcoma	2 (4%)			1 (2%)
Thymus	(44)			(47)
Squamous cell carcinoma, metastatic, lung				1 (2%)
Integumentary System				
Mammary gland	(49)			(50)
Adenoma	1 (2%)			
Fibroadenoma	1 (2%)			3 (6%)
Fibroma	3 (6%)			1 (2%)
Histiocytic sarcoma	1 (2%)			
Skin, control	(52)	(52)	(55)	(56)
Basal cell adenoma			1 (2%)	
Basosquamous tumor benign			1 (2%)	
Histiocytic sarcoma	1 (2%)			
Keratoacanthoma	1 (2%)		3 (5%)	
Squamous cell papilloma	1 (2%)			1 (2%)
Sebaceous gland, lip, carcinoma	1 (2%)			
Subcutaneous tissue, fibroma			1 (2%)	1 (2%)
Subcutaneous tissue, fibrosarcoma		1 (2%)		
Subcutaneous tissue, fibrous histiocytoma		1 (2%)		
Subcutaneous tissue, histiocytic sarcoma				1 (2%)
Subcutaneous tissue, neurofibroma	1 (2%)			
Skin, site of application-no mass	(52)	(52)	(55)	(56)
Subcutaneous tissue, histiocytic sarcoma	1 (2%)	1 (2%)		1 (2%)
Skin, site of application-mass	(5)		(3)	(4)
Keratoacanthoma			1 (33%)	1 (25%)
Sebaceous gland, carcinoma			1 (33%)	
Subcutaneous tissue, histiocytic sarcoma	1 (20%)		1 (33%)	

TABLE A1

Summary of the Incidence of Neoplasms in Male Rats in the 2-Year Dermal Study of Benzethonium Chloride (continued)

	Vehicle Control	0.15 mg/kg	0.5 mg/kg	1.5 mg/kg
2-Year Study (continued)				
Musculoskeletal System				
Bone	(52)			(56)
Maxilla, osteosarcoma	1 (2%)			
Nervous System				
Brain	(52)			(56)
Astrocytoma NOS				1 (2%)
Respiratory System				
Lung	(51)			(56)
Histiocytic sarcoma	1 (2%)			
Squamous cell carcinoma				1 (2%)
Special Senses System				
Ear	(3)			
Squamous cell papilloma	3 (100%)			
Zymbal's gland	(2)			
Carcinoma	2 (100%)			
Urinary System				
Kidney	(52)			(56)
Transitional epithelium, carcinoma	1 (2%)			
Urinary bladder	(52)			(56)
Transitional epithelium, papilloma				1 (2%)
Systemic Lesions				
Multiple organs ^b	(52)	(52)	(55)	(56)
Histiocytic sarcoma	3 (6%)	1 (2%)	1 (2%)	3 (5%)
Leukemia mononuclear	23 (44%)			30 (54%)
Mesothelioma benign				1 (2%)
Mesothelioma malignant	1 (2%)			1 (2%)

TABLE A1

Summary of the Incidence of Neoplasms in Male Rats in the 2-Year Dermal Study of Benzethonium Chloride (continued)

	Vehicle Control	0.15 mg/kg	0.5 mg/kg	1.5 mg/kg
2-Year Study (continued)				
Neoplasm Summary				
Total animals with primary neoplasms ^c				
15-Month interim evaluation	5			3
2-Year study	52	3	8	54
Total primary neoplasms				
15-Month interim evaluation	7			4
2-Year study	142	3	9	131
Total animals with benign neoplasms				
15-Month interim evaluation	5			3
2-Year study	50		7	51
Total benign neoplasms				
15-Month interim evaluation	7			4
2-Year study	104		7	93
Total animals with malignant neoplasms				
2-Year study	30	3	2	33
Total malignant neoplasms				
2-Year study	38	3	2	37
Total animals with metastatic neoplasms				
2-Year study				1
Total metastatic neoplasms				
2-Year study				3
Total animals with uncertain neoplasms				
benign or malignant				
2-Year study				1
Total uncertain neoplasms				
2-Year study				1

^a Number of animals examined microscopically at the site and the number of animals with neoplasm^b Number of animals with any tissue examined microscopically^c Primary neoplasms: all neoplasms except metastatic neoplasms

TABLE A2

[illegible]

X: Lesion present
Blank: Not examined

[illegible]

TABLE A2
Individual Animal Tumor Pathology of Male Rats in the 2-Year Dermal Study
of Benzethonium Chloride: Vehicle Control (continued)

[illegible]

TABLE A2
Individual Animal Tumor Pathology of Male Rats in the 2-Year Dermal Study
of Benzethonium Chloride: Vehicle Control (continued)

[illegible]

TABLE A2

	4	4	5	5	5	5	5	5	5	5	6	6	6	6	6	6	6	6	6	6	6	6	6	6
Number of Days on Study	0	3	1	3	4	5	5	6	8	8	9	0	0	0	0	1	3	3	3	4	4	4	5	5
	6	7	3	3	9	1	6	4	3	5	2	3	6	6	6	2	2	4	5	7	7	8	0	3
	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Carcass ID Number	3	4	0	0	4	4	1	6	5	5	3	0	0	1	1	5	3	5	4	1	5	5	0	1
	2	6	6	4	1	9	8	0	6	4	6	5	9	3	6	0	4	3	8	9	8	5	2	1
Musculoskeletal System																								
Bone	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Maxilla, osteosarcoma																			X					
Nervous System																								
Brain	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Peripheral nerve												+	+			+								
Spinal cord												+	+			+								
Respiratory System																								
Lung	+	+	+	+	+	M	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Histiocytic sarcoma					X																			
Nose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Trachea	+	+	+	+	+	M	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Special Senses System																								
Ear																								
Squamous cell papilloma																								
Eye																								
Zymbal's gland															+									
Carcinoma															X									
Urinary System																								
Kidney	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Transitional epithelium, carcinoma									X															
Urinary bladder	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Systemic Lesions																								
Multiple organs	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Histiocytic sarcoma			X		X				X															
Leukemia mononuclear					X	X	X		X	X	X	X		X		X		X	X	X		X	X	
Mesothelioma malignant																								

[illegible]

Individual Animal Tumor Pathology of Male Rats in the 2-Year Dermal Study of Benzethonium Chloride: 0.15 mg/kg

Board Draft

Individual Animal Tumor Pathology of Male Rats in the 2-Year Dermal Study of Benzethonium Chloride: 0.15 mg/kg
(continued)

[illegible]

TABLE A2

Individual Animal Tumor Pathology of Male Rats in the 2-Year Dermal Study of Benzethonium Chloride: 0.5 mg/kg

Number of Days on Study	0 1 3 4 4 4 5 5 5 5 5 5 5 5 5 5 5 5 6 6 6 6 6 6 6 6 6 6 6 6
	2 9 6 1 3 8 0 0 1 2 2 3 5 7 8 8 9 0 1 1 2 2 3 3 3 3 3 4
	5 9 7 6 7 3 0 5 1 3 6 7 6 5 7 9 9 1 3 3 1 6 2 2 5 5 3
Carcass ID Number	1 1
	6 6 4 2 3 4 2 5 3 3 6 6 2 7 4 4 3 6 5 8 6 5 3 7 5 5 2
	0 5 0 7 3 5 2 6 9 0 3 1 1 6 6 9 1 7 9 0 6 7 7 2 2 3 6
Alimentary System	
None	
Cardiovascular System	
None	
Endocrine System	
None	
General Body System	
None	
Genital System	
None	
Hematopoietic System	
None	
Integumentary System	
Skin, control	+ +
Basal cell adenoma	
Basosquamous tumor benign	
Keratoacanthoma	
Subcutaneous tissue, fibroma	
Skin, site of application-no mass	+ +
Skin, site of application-mass	
Keratoacanthoma	
Sebaceous gland, carcinoma	
Subcutaneous tissue, histiocytic sarcoma	
Musculoskeletal System	
None	
Nervous System	
None	
Respiratory System	
None	
Special Senses System	
None	
Urinary System	
None	
Systemic Lesions	
Multiple organs	+ +
Histiocytic sarcoma	

[illegible]

TABLE A2
Individual Animal Tumor Pathology of Male Rats in the 2-Year Dermal Study of Benzethonium Chloride: 1.5 mg/kg

TABLE A2
Individual Animal Tumor Pathology of Male Rats in the 2-Year Dermal Study of Benzethonium Chloride: 1.5 mg/kg
(continued)

[illegible]

Individual Animal Tumor Pathology of Male Rats in the 2-Year Dermal Study of Benzethonium Chloride: 1.5 mg/kg
(continued)

[illegible]

Individual Animal Tumor Pathology of Male Rats in the 2-Year Dermal Study of Benzethonium Chloride: 1.5 mg/kg
(continued)

	1	2	3	3	4	4	4	5	5	5	5	5	5	5	5	6	6	6	6	6	6	6	6	6	6	6	6	
Number of Days on Study	7	9	6	9	0	2	6	3	3	4	5	5	5	5	6	9	0	0	1	1	1	2	3	3	3	4	4	5
	0	1	4	6	8	5	5	3	7	9	1	6	7	2	4	5	9	3	3	9	6	5	9	9	3	4	9	5
Carcass ID Number	2	2	2	2	2	2	2	1	1	1	2	2	1	1	1	2	2	1	2	2	1	2	1	2	2	2	2	2
	0	0	0	3	0	4	0	8	9	8	2	2	9	8	9	0	1	9	1	1	8	3	8	0	2	3	1	1
	7	3	1	7	0	0	5	5	1	6	0	5	3	7	9	9	2	0	5	7	8	3	3	6	3	4	1	4
Respiratory System																												
Lung	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
Squamous cell carcinoma															X													
Nose	+	+	+	+	+	M	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
Trachea	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
Special Senses System																												
Eye																												
Urinary System																												
Kidney	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
Urinary bladder	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
Transitional epithelium, papilloma																												
Systemic Lesions																												
Multiple organs	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
Histiocytic sarcoma														X					X									
Leukemia mononuclear					X	X		X	X	X	X	X	X	X	X	X	X	X	X	X	X						X	
Mesothelioma benign				X																								
Mesothelioma malignant																								X				

TABLE A3
Statistical Analysis of Primary Neoplasms in Male Rats in the 2-Year Dermal Study of Benzethonium Chloride

	Vehicle Control	0.15 mg/kg	0.5 mg/kg	1.5 mg/kg
Adrenal Medulla: Benign Pheochromocytoma				
Overall rate ^a	5/52 (10%)	— ^e	—	7/56 (13%)
Adjusted rate ^b	20.3%			28.7%
Terminal rate ^c	2/15 (13%)			3/16 (19%)
First incidence (days)	556			619
Life table test ^d				P=0.401
Logistic regression test ^d				P=0.387
Fisher exact test ^d				P=0.434
Ear: Squamous Cell Papilloma				
Overall rate	3/52 (6%)	—	—	0/56 (0%)
Adjusted rate	17.9%			0.0%
Terminal rate	2/15 (13%)			0/16 (0%)
First incidence (days)	714			— ^f
Life table test				P=0.117N
Logistic regression test				P=0.115N
Fisher exact test				P=0.108N
Mammary Gland: Fibroma				
Overall rate	3/52 (6%)	—	—	1/56 (2%)
Adjusted rate	16.1%			4.3%
Terminal rate	2/15 (13%)			0/16 (0%)
First incidence (days)	648			683
Life table test				P=0.302N
Logistic regression test				P=0.306N
Fisher exact test				P=0.281N
Mammary Gland: Fibroadenoma				
Overall rate	1/52 (2%)	—	—	3/56 (5%)
Adjusted rate	2.5%			17.4%
Terminal rate	0/15 (0%)			2/16 (13%)
First incidence (days)	606			722
Life table test				P=0.329
Logistic regression test				P=0.301
Fisher exact test				P=0.338
Mammary Gland: Fibroma, Fibroadenoma, or Adenoma				
Overall rate	5/52 (10%)	—	—	4/56 (7%)
Adjusted rate	24.5%			21.0%
Terminal rate	3/15 (20%)			2/16 (13%)
First incidence (days)	606			683
Life table test				P=0.475N
Logistic regression test				P=0.505N
Fisher exact test				P=0.453N

TABLE A3

Statistical Analysis of Primary Neoplasms in Male Rats in the 2-Year Dermal Study of Benzethonium Chloride
(continued)

	Vehicle Control	0.15 mg/kg	0.5 mg/kg	1.5 mg/kg
Pancreatic Islets: Adenoma				
Overall rate	1/52 (2%)	—	—	3/56 (5%)
Adjusted rate	2.9%			18.8%
Terminal rate	0/15 (0%)			3/16 (19%)
First incidence (days)	634			729 (T)
Life table test				P=0.321
Logistic regression test				P=0.333
Fisher exact test				P=0.338
Pituitary Gland (Pars Distalis or Unspecified Site): Adenoma				
Overall rate	31/52 (60%)	—	—	23/56 (41%)
Adjusted rate	84.4%			65.0%
Terminal rate	10/15 (67%)			6/16 (38%)
First incidence (days)	406			408
Life table test				P=0.136N
Logistic regression test				P=0.085N
Fisher exact test				P=0.041N
Skin: Keratoacanthoma				
Overall rate	1/52 (2%)	0/52 (0%)	3/55 (5%)	0/56 (0%)
Adjusted rate	6.7%	0.0%	26.5%	0.0%
Terminal rate	1/15 (7%)	0/11 (0%)	2/9 (22%)	0/16 (0%)
First incidence (days)	729 (T)	—	676	—
Life table test	P=0.394N	P=0.562N	P=0.155	P=0.487N
Logistic regression test	P=0.416N	P=0.562N	P=0.187	P=0.487N
Cochran-Armitage test	P=0.425N			
Fisher exact test		P=0.500N	P=0.330	P=0.481N
Testes: Adenoma				
Overall rate	42/52 (81%)	—	—	40/56 (71%)
Adjusted rate	100.0%			97.3%
Terminal rate	15/15 (100%)			5/16 (94%)
First incidence (days)	513			465
Life table test				P=0.389N
Logistic regression test				P=0.422N
Fisher exact test				P=0.182N
Thyroid Gland (C-cell): Adenoma				
Overall rate	7/51 (14%)	—	—	7/56 (13%)
Adjusted rate	33.5%			31.2%
Terminal rate	4/15 (27%)			4/16 (25%)
First incidence (days)	585			465
Life table test				P=0.584N
Logistic regression test				P=0.584N
Fisher exact test				P=0.538N
All Organs: Histiocytic Sarcoma				
Overall rate	3/52 (6%)	—	—	3/56 (5%)
Adjusted rate	6.2%			10.9%
Terminal rate	0/15 (0%)			1/16 (6%)
First incidence (days)	513			557
Life table test				P=0.657
Logistic regression test				P=0.560N
Fisher exact test				P=0.625N

TABLE A3

Statistical Analysis of Primary Neoplasms in Male Rats in the 2-Year Dermal Study of Benzethonium Chloride

(continued)

	Vehicle Control	0.15 mg/kg	0.5 mg/kg	1.5 mg/kg
All Organs: Mononuclear Cell Leukemia				
Overall rate	23/52 (44%)	—	—	30/56 (54%)
Adjusted rate	61.7%			82.0%
Terminal rate	5/15 (33%)			11/16 (69%)
First incidence (days)	549			396
Life table test				P=0.214
Logistic regression test				P=0.209
Fisher exact test				P=0.218
All Organs: Benign Neoplasms				
Overall rate	50/52 (96%)	—	—	52/56 (93%)
Adjusted rate	100.0%			100.0%
Terminal rate	15/15 (100%)			16/16 (100%)
First incidence (days)	406			364
Life table test				P=0.501
Logistic regression test				P=0.649
Fisher exact test				P=0.375N
All Organs: Malignant Neoplasms				
Overall rate	30/52 (58%)	—	—	33/56 (59%)
Adjusted rate	70.8%			83.4%
Terminal rate	6/15 (40%)			11/16 (69%)
First incidence (days)	437			396
Life table test				P=0.424
Logistic regression test				P=0.559
Fisher exact test				P=0.526
All Organs: Benign or Malignant Neoplasms				
Overall rate	52/52 (100%)	—	—	54/56 (96%)
Adjusted rate	100.0%			100.0%
Terminal rate	15/15 (100%)			16/16 (100%)
First incidence (days)	406			364
Life table test				P=0.501
Logistic regression test				— ^g
Fisher exact test				P=0.267N

(T)Terminal sacrifice

^a Number of neoplasm-bearing animals/number of animals examined. Denominator is number of animals examined microscopically for adrenal gland, bone marrow, brain, epididymis, heart, kidney, larynx, liver, lung, nose, pancreas, parathyroid gland, pituitary gland, preputial gland, prostate gland, salivary gland, spleen, testes, thyroid gland, and urinary bladder; for other tissues, denominator is number of animals necropsied.

^b Kaplan-Meier estimated neoplasm incidence at the end of the study after adjustment for intercurrent mortality

^c Observed incidence at terminal kill

^d Beneath the dosed group incidence are the P values corresponding to pairwise comparisons between the controls and that dosed group. The life table test regards neoplasms in animals dying prior to terminal kill as being (directly or indirectly) the cause of death. The logistic regression test regards these lesions as nonfatal. The Cochran-Armitage and Fisher exact tests compare directly the overall incidence rates. For all tests, a negative trend or a lower incidence in a dose group is indicated by N.

^e Organ was not examined at this dose level

^f Not applicable; no neoplasms in animal group

^g Value of statistic cannot be computed.

TABLE A4
Summary of the Incidence of Nonneoplastic Lesions in Male Rats in the 2-Year Dermal Study of Benzethonium Chloride^a

	Vehicle Control	0.15 mg/kg	0.5 mg/kg	1.5 mg/kg
Disposition Summary				
Animals initially in study	60	60	60	60
<i>15-Month interim evaluation</i>	8	8	5	4
Early deaths				
Accidental death			1	
Moribund	25	34	34	25
Natural deaths	12	7	11	15
Survivors				
Died last week of study			1	
Terminal sacrifice	15	11	8	16
Animals examined microscopically	60	60	60	60
15-Month Interim Evaluation				
Alimentary System				
Intestine small, jejunum	(1)			
Inflammation, chronic	1 (100%)			
Liver	(8)			(4)
Hepatodiaphragmatic nodule	3 (38%)			
Inflammation, chronic active	3 (38%)			2 (50%)
Bile duct, hyperplasia	3 (38%)			2 (50%)
Hepatocyte, vacuolization cytoplasmic	5 (63%)			4 (100%)
Pancreas	(8)			(4)
Acinus, atrophy	5 (63%)			2 (50%)
Cardiovascular System				
Heart	(8)			(4)
Degeneration, chronic	8 (100%)			3 (75%)
Endocrine System				
Adrenal cortex	(1)			
Hyperplasia	1 (100%)			
Pituitary gland	(8)			(4)
Pars distalis, cyst	1 (13%)			
Pars distalis, hyperplasia				1 (25%)
Genital System				
Epididymis	(8)			(4)
Aspermia				1 (25%)
Preputial gland	(8)			(4)
Inflammation, chronic active	5 (63%)			2 (50%)
Prostate	(8)			(4)
Inflammation, chronic active	4 (50%)			2 (50%)
Testes	(8)			(4)
Germinal epithelium, degeneration				1 (25%)
Interstitial cell, hyperplasia	8 (100%)			3 (75%)

TABLE A4

Summary of the Incidence of Nonneoplastic Lesions in Male Rats in the 2-Year Dermal Study of Benzethonium Chloride (continued)

	Vehicle Control	0.15 mg/kg	0.5 mg/kg	1.5 mg/kg
15-Month Interim Evaluation (continued)				
Hematopoietic System				
Lymph node	(1)			
Pancreatic, angiectasis	1 (100%)			
Lymph node, mesenteric	(1)			
Angiectasis	1 (100%)			
Spleen	(8)			(4)
Fibrosis	1 (13%)			
Thymus	(8)			(4)
Depletion lymphoid	8 (100%)			3 (75%)
Integumentary System				
Mammary gland	(6)			(3)
Hyperplasia, cystic	3 (50%)			1 (33%)
Skin, control	(8)	(8)	(5)	(4)
Skin, site of application-no mass	(8)	(8)	(5)	(4)
Epithelial hyperplasia			3 (60%)	4 (100%)
Ulcer				1 (25%)
Sebaceous gland, hyperplasia			2 (40%)	2 (50%)
Respiratory System				
Lung	(8)			(4)
Alveolar epithelium, hyperplasia	1 (13%)			
Alveolar epithelium, hyperplasia, focal	1 (13%)			
Nose	(8)			(4)
Submucosa, inflammation, acute	1 (13%)			
Urinary System				
Kidney	(8)			(4)
Nephropathy, chronic	8 (100%)			4 (100%)
Cortex, cyst	1 (13%)			
Cortex, renal tubule, mineralization	1 (13%)			
Systems Examined With No Lesions Observed				
General Body System				
Musculoskeletal System				
Nervous System				
Special Senses System				

TABLE A4

Summary of the Incidence of Nonneoplastic Lesions in Male Rats in the 2-Year Dermal Study of Benzethonium Chloride (continued)

	Vehicle Control	0.15 mg/kg	0.5 mg/kg	1.5 mg/kg
2-Year Study				
Alimentary System				
Intestine small, duodenum				(3)
Dysplasia				1 (33%)
Ulcer				1 (33%)
Liver	(52)			(56)
Basophilic focus	6 (12%)			7 (13%)
Clear cell focus	3 (6%)			3 (5%)
Eosinophilic focus	4 (8%)			3 (5%)
Hematopoietic cell proliferation	1 (2%)			
Hepatodiaphragmatic node	3 (6%)			4 (7%)
Inflammation, chronic active				1 (2%)
Inflammation, granulomatous	2 (4%)			3 (5%)
Bile duct, cyst				1 (2%)
Bile duct, hyperplasia	34 (65%)			36 (64%)
Hepatocyte, degeneration, cystic	4 (8%)			9 (16%)
Hepatocyte, hyperplasia				1 (2%)
Hepatocyte, vacuolization cytoplasmic	17 (33%)			17 (30%)
Mesentery	(8)			(8)
Artery, inflammation, chronic active	1 (13%)			1 (13%)
Fat, hemorrhage	1 (13%)			
Fat, inflammation, chronic active	3 (38%)			6 (75%)
Pancreas	(52)			(56)
Acinus, atrophy	18 (35%)			24 (43%)
Artery, inflammation, chronic active	1 (2%)			1 (2%)
Stomach, forestomach	(9)			(13)
Acanthosis	4 (44%)			2 (15%)
Inflammation, chronic active	1 (11%)			2 (15%)
Mineralization	1 (11%)			2 (15%)
Ulcer	5 (56%)			8 (62%)
Stomach, glandular	(13)			(11)
Dysplasia	2 (15%)			
Hyperplasia				1 (9%)
Mineralization	5 (38%)			3 (27%)
Necrosis	1 (8%)			2 (18%)
Pigmentation, hemosiderin	1 (8%)			
Ulcer	4 (31%)			5 (45%)
Tongue	(1)			
Inflammation, chronic active	1 (100%)			
Tooth	(2)			
Peridontal tissue, inflammation, chronic active	1 (50%)			
Cardiovascular System				
Blood vessel	(1)			(1)
Aorta, mineralization	1 (100%)			1 (100%)
Heart	(51)			(56)
Degeneration, chronic	43 (84%)			47 (84%)
Mineralization	1 (2%)			2 (4%)
Atrium, thrombosis	6 (12%)			4 (7%)

TABLE A4
Summary of the Incidence of Nonneoplastic Lesions in Male Rats in the 2-Year Dermal Study
of Benzethonium Chloride (continued)

	Vehicle Control	0.15 mg/kg	0.5 mg/kg	1.5 mg/kg
2-Year Study (continued)				
Endocrine System				
Adrenal cortex	(28)			(24)
Hyperplasia	12 (43%)			6 (25%)
Hypertrophy	2 (7%)			
Vacuolization cytoplasmic	22 (79%)			20 (83%)
Adrenal medulla	(27)			(29)
Hyperplasia	24 (89%)			21 (72%)
Islets, pancreatic	(52)			(56)
Hyperplasia	1 (2%)			1 (2%)
Parathyroid gland	(50)			(54)
Hyperplasia	37 (74%)			39 (72%)
Pituitary gland	(52)			(56)
Pars distalis, cyst	6 (12%)			
Pars distalis, hemorrhage				2 (4%)
Pars distalis, hyperplasia	13 (25%)			10 (18%)
Pars distalis, vacuolization cytoplasmic				2 (4%)
Thyroid gland	(51)			(56)
Ultimobranchial cyst				1 (2%)
C-cell, hyperplasia	12 (24%)			11 (20%)
Follicle, cyst				1 (2%)
Follicular cell, hyperplasia	1 (2%)			
General Body System				
None				
Genital System				
Epididymis	(52)			(56)
Aspermia				1 (2%)
Inflammation, granulomatous				1 (2%)
Preputial gland	(52)			(56)
Inflammation, chronic active	41 (79%)			36 (64%)
Duct, ectasia	1 (2%)			2 (4%)
Prostate	(52)			(56)
Hyperplasia				1 (2%)
Inflammation, chronic active	26 (50%)			24 (43%)
Inflammation, granulomatous				1 (2%)
Epithelium, hyperplasia	1 (2%)			
Seminal vesicle	(52)			(56)
Atrophy				1 (2%)
Inflammation, chronic active	1 (2%)			2 (4%)
Mineralization	1 (2%)			
Testes	(52)			(56)
Cyst				1 (2%)
Germinal epithelium, degeneration	16 (31%)			16 (29%)
Interstitial cell, hyperplasia	9 (17%)			10 (18%)

TABLE A4
Summary of the Incidence of Nonneoplastic Lesions in Male Rats in the 2-Year Dermal Study
of Benzethonium Chloride (continued)

	Vehicle Control	0.15 mg/kg	0.5 mg/kg	1.5 mg/kg
2-Year Study (continued)				
Hematopoietic System				
Bone marrow	(52)			(56)
Femoral, myelofibrosis	4 (8%)			4 (7%)
Lymph node	(7)			(14)
Mediastinal, congestion				1 (7%)
Mediastinal, inflammation, chronic active				1 (7%)
Lymph node, mandibular	(13)			(17)
Hyperplasia, lymphoid	1 (8%)			
Infiltration cellular, plasma cell				1 (6%)
Lymph node, mesenteric	(8)			(14)
Hyperplasia, lymphoid				1 (7%)
Inflammation, chronic active				1 (7%)
Spleen	(52)			(56)
Fibrosis	13 (25%)			7 (13%)
Infarct				1 (2%)
Thrombosis				1 (2%)
Capsule, thrombosis				1 (2%)
Thymus	(44)			(47)
Depletion lymphoid	1 (2%)			4 (9%)
Integumentary System				
Mammary gland	(49)			(50)
Hyperplasia, cystic	47 (96%)			48 (96%)
Duct, cyst				1 (2%)
Skin, control	(52)	(52)	(55)	(56)
Cyst epithelial inclusion				1 (2%)
Dermis, inflammation, chronic		1 (2%)		
Epithelial hyperplasia	7 (13%)	5 (10%)	2 (4%)	3 (5%)
Hyperplasia, basal cell			1 (2%)	
Ulcer				1 (2%)
Skin, site of application-no mass	(52)	(52)	(55)	(56)
Cyst				1 (2%)
Epithelial hyperplasia	1 (2%)		4 (7%)	12 (21%)
Sebaceous gland, hyperplasia			2 (4%)	2 (4%)
Skin, site of application-mass	(5)		(3)	(4)
Cyst epithelial inclusion	1 (20%)			2 (50%)
Dermis, fibrosis	3 (60%)			1 (25%)
Dermis, mineralization	2 (40%)			
Musculoskeletal System				
Bone	(52)			(56)
Tarsal, fracture				1 (2%)

TABLE A4
Summary of the Incidence of Nonneoplastic Lesions in Male Rats in the 2-Year Dermal Study
of Benzethonium Chloride (continued)

	Vehicle Control	0.15 mg/kg	0.5 mg/kg	1.5 mg/kg
2-Year Study (continued)				
Nervous System				
Brain	(52)			(56)
Compression	14 (27%)			13 (23%)
Hemorrhage	1 (2%)			
Hydrocephalus	10 (19%)			6 (11%)
Respiratory System				
Lung	(51)			(56)
Inflammation, chronic active	3 (6%)			2 (4%)
Inflammation, granulomatous				1 (2%)
Inflammation, suppurative	1 (2%)			
Mineralization	1 (2%)			2 (4%)
Nose	(52)			(55)
Lumen, hemorrhage				1 (2%)
Mucosa, inflammation, chronic active	6 (12%)			6 (11%)
Mucosa, ulcer				1 (2%)
Sinus, foreign body	6 (12%)			2 (4%)
Trachea	(51)			(56)
Submucosa, inflammation, chronic				1 (2%)
Special Senses System				
Eye	(2)			(3)
Lens, cataract	2 (100%)			2 (67%)
Retina, atrophy	2 (100%)			2 (67%)
Urinary System				
Kidney	(52)			(56)
Cyst				1 (2%)
Nephropathy, chronic	52 (100%)			56 (100%)
Urinary bladder	(52)			(56)
Inflammation, chronic active	1 (2%)			
Transitional epithelium, hyperplasia	1 (2%)			

^a Number of animals examined microscopically at the site and the number of animals with lesion

APPENDIX B
SUMMARY OF LESIONS IN FEMALE RATS
IN THE 2-YEAR DERMAL STUDY
OF BENZETHONIUM CHLORIDE

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TABLE B1
Summary of the Incidence of Neoplasms in Female Rats in the 2-Year Dermal Study of Benzethonium Chloride¹

	Vehicle Control	0.15 mg/kg	0.5 mg/kg	1.5 mg/kg
Disposition Summary				
Animals initially in study	60	60	60	60
15-Month interim evaluation	9	7	9	7
Early deaths				
Moribund	13	11	13	13
Natural deaths	14	9	12	16
Survivors				
Terminal sacrifice	24	33	26	24
Animals examined microscopically	60	60	60	60
15-Month Interim Evaluation				
Alimentary System				
Mesentery	(1)			(1)
Fat, liposarcoma	1 (100%)			
Endocrine System				
Pituitary gland	(9)			(7)
Pars distalis, adenoma	1 (11%)			1 (14%)
Genital System				
Clitoral gland	(9)			(7)
Adenoma	1 (11%)			
Integumentary System				
Skin, control	(9)	(7)	(9)	(7)
Subcutaneous tissue, lipoma	1 (11%)			
Skin, site of application	(9)	(7)	(9)	(7)
Systems Examined With No Neoplasms Observed				
Cardiovascular System				
General Body System				
Hematopoietic System				
Musculoskeletal System				
Nervous System				
Respiratory System				
Special Senses System				
Urinary System				
2-Year Study				
Alimentary System				
Intestine small, jejunum	(1)			
Liver	(51)			(53)
Pancreas	(51)			(53)
Salivary glands	(51)			(53)

TABLE B1

Summary of the Incidence of Neoplasms in Female Rats in the 2-Year Dermal Study of Benzethonium Chloride
(continued)

	Vehicle Control	0.15 mg/kg	0.5 mg/kg	1.5 mg/kg
2-Year Study (continued)				
Cardiovascular System				
None				
Endocrine System				
Adrenal cortex	(19)			(21)
Adenoma				1 (5%)
Adrenal medulla	(10)			(2)
Pheochromocytoma benign	3 (30%)			
Bilateral, pheochromocytoma benign				1 (50%)
Islets, pancreatic	(51)			(53)
Carcinoma				1 (2%)
Pituitary gland	(51)			(53)
Pars distalis, adenoma	27 (53%)			27 (51%)
Pars distalis, adenoma, multiple	1 (2%)			
Pars distalis, carcinoma	2 (4%)			2 (4%)
Thyroid gland	(51)			(53)
Bilateral, C-cell, adenoma	1 (2%)			
C-cell, adenoma	10 (20%)			5 (9%)
C-cell, carcinoma	1 (2%)			
General Body System				
None				
Genital System				
Clitoral gland	(50)			(52)
Adenoma	3 (6%)			4 (8%)
Carcinoma				2 (4%)
Ovary	(51)			(53)
Uterus	(51)			(53)
Polyp stromal	3 (6%)			7 (13%)
Hematopoietic System				
Bone marrow	(51)			(52)
Lymph node	(5)			(1)
Lymph node, mandibular	(7)			(3)
Osteosarcoma, metastatic, bone				1 (33%)
Lymph node, mesenteric	(5)			(3)
Spleen	(51)			(53)
Thymus	(46)			(50)
Integumentary System				
Mammary gland	(51)			(53)
Adenoma	2 (4%)			2 (4%)
Adenoma, multiple	1 (2%)			
Carcinoma				1 (2%)
Fibroadenoma	14 (27%)			12 (23%)
Fibroadenoma, multiple	3 (6%)			5 (9%)
Fibroma				1 (2%)

TABLE B1
Summary of the Incidence of Neoplasms in Female Rats in the 2-Year Dermal Study of Benzethonium Chloride
 (continued)

	Vehicle Control	0.15 mg/kg	0.5 mg/kg	1.5 mg/kg
2-Year Study (continued)				
Integumentary System (continued)				
Skin, control	(51)	(53)	(51)	(53)
Basosquamous tumor malignant				1 (2%)
Basosquamous tumor benign	1 (2%)			
Trichoepithelioma			1 (2%)	
Subcutaneous tissue, lipoma				1 (2%)
Subcutaneous tissue, neurofibrosarcoma			1 (2%)	
Skin, site of application-no mass	(51)	(53)	(51)	(53)
Musculoskeletal System				
Bone	(51)			(53)
Mandible, osteosarcoma				1 (2%)
Nervous System				
Brain	(51)			(53)
Carcinoma, metastatic, pituitary gland	2 (4%)			2 (4%)
Respiratory System				
Lung	(51)			(53)
Carcinoma, metastatic, thyroid gland	1 (2%)			
Nose	(51)			(53)
Nares, squamous cell papilloma	1 (2%)			
Vomeronasal organ, squamous cell carcinoma	1 (2%)			
Special Senses System				
None				
Urinary System				
Kidney	(51)			(53)
Urinary bladder	(51)			(53)
Systemic Lesions				
Multiple organs ^b	(51)	(53)	(51)	(53)
Leukemia mononuclear	18 (35%)			18 (34%)

TABLE B1

Summary of the Incidence of Neoplasms in Female Rats in the 2-Year Dermal Study of Benzethonium Chloride
(continued)

	Vehicle Control	0.15 mg/kg	0.5 mg/kg	1.5 mg/kg
2-Year Study (continued)				
Cardiovascular System				
None				
Endocrine System				
Adrenal cortex	(19)			(21)
Adenoma				1 (5%)
Adrenal medulla	(10)			(2)
Pheochromocytoma benign	3 (30%)			
Bilateral, pheochromocytoma benign				1 (50%)
Islets, pancreatic	(51)			(53)
Carcinoma				1 (2%)
Pituitary gland	(51)			(53)
Pars distalis, adenoma	27 (53%)			27 (51%)
Pars distalis, adenoma, multiple	1 (2%)			
Pars distalis, carcinoma	2 (4%)			2 (4%)
Thyroid gland	(51)			(53)
Bilateral, C-cell, adenoma	1 (2%)			
C-cell, adenoma	10 (20%)			5 (9%)
C-cell, carcinoma	1 (2%)			
General Body System				
None				
Genital System				
Clitoral gland	(50)			(52)
Adenoma	3 (6%)			4 (8%)
Carcinoma				2 (4%)
Ovary	(51)			(53)
Uterus	(51)			(53)
Polyp stromal	3 (6%)			7 (13%)
Hematopoietic System				
Bone marrow	(51)			(52)
Lymph node	(5)			(1)
Lymph node, mandibular	(7)			(3)
Osteosarcoma, metastatic, bone				1 (33%)
Lymph node, mesenteric	(5)			(3)
Spleen	(51)			(53)
Thymus	(46)			(50)
Integumentary System				
Mammary gland	(51)			(53)
Adenoma	2 (4%)			2 (4%)
Adenoma, multiple	1 (2%)			
Carcinoma				1 (2%)
Fibroadenoma	14 (27%)			12 (23%)
Fibroadenoma, multiple	3 (6%)			5 (9%)
Fibroma				1 (2%)

TABLE B1
Summary of the Incidence of Neoplasms in Female Rats in the 2-Year Dermal Study of Benzethonium Chloride
 (continued)

	Vehicle Control	0.15 mg/kg	0.5 mg/kg	1.5 mg/kg
Neoplasm Summary				
Total animals with primary neoplasms ^c				
15-Month interim evaluation	3			1
2-Year study	43		2	49
Total primary neoplasms				
15-Month interim evaluation	4			1
2-Year study	92		2	92
Total animals with benign neoplasms				
15-Month interim evaluation	3			1
2-Year study	38		1	41
Total benign neoplasms				
15-Month interim evaluation	3			1
2-Year study	70		1	66
Total animals with malignant neoplasms				
15-Month interim evaluation	1			
2-Year study	22		1	21
Total malignant neoplasms				
15-Month interim evaluation	1			
2-Year study	22		1	26
Total animals with metastatic neoplasms				
2-Year study	3			3
Total metastatic neoplasms				
2-Year study	3			3

^a Number of animals examined microscopically at the site and the number of animals with neoplasm

^b Number of animals with any tissue examined microscopically

^c Primary neoplasms: all neoplasms except metastatic neoplasms

TABLE B2
Individual Animal Tumor Pathology of Female Rats in the 2-Year Dermal Study
of Benzethonium Chloride: Vehicle Control

	1	4	5	5	5	5	5	6	6	6	6	6	6	6	6	6	6	6	6	7	7	7	7	7	
Number of Days on Study	7	8	1	1	2	5	7	9	0	1	1	3	4	4	4	4	4	6	9	0	1	1	2	2	
	6	5	5	9	6	4	2	5	3	0	2	3	2	2	4	5	8	9	7	6	9	8	9	0	2
Carcass ID Number	2	2	2	2	2	2	2	2	2	3	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2
	5	7	9	5	8	7	4	7	9	6	0	7	6	9	6	7	4	5	8	4	9	9	8	7	9
	7	5	7	1	1	2	7	6	4	1	0	0	3	6	2	9	3	9	3	9	9	1	4	3	5
Alimentary System																									
Esophagus	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Intestine small, duodenum									+													+			
Intestine small, jejunum												+													
Liver	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Mesentery								+															+		
Pancreas	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Salivary glands	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Stomach, forestomach	+		+					+													+				
Stomach, glandular	+																								
Cardiovascular System																									
Heart	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Endocrine System																									
Adrenal cortex		+					+		+		+	+				+	+			+	+	+		+	
Adrenal medulla											+	+				+						+			
Pheochromocytoma benign																									
Islets, pancreatic	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Parathyroid gland	+	+	+	+	+	+	+	+	+	+	M	+	+	+	+	+	M	+	+	+	M	+	+	+	+
Pituitary gland	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Pars distalis, adenoma				X	X	X	X	X							X	X	X	X	X	X					X
Pars distalis, adenoma, multiple																									
Pars distalis, carcinoma																									
Thyroid gland	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Bilateral, C-cell, adenoma																									
C-cell, adenoma				X				X										X				X	X		
C-cell, carcinoma																									
General Body System																									
None																									
Genital System																									
Clitoral gland	+	+	+	+	+	+	+	+	+	+	+	+	+	M	+	+	+	+	+	+	+	+	+	+	+
Adenoma								X																	
Ovary	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Uterus	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Polyp stromal			X																						
Vagina																							+		
Hematopoietic System																									
Blood									+												+				
Bone marrow	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Lymph node		+								+	+											+			
Lymph node, mandibular	+									+	+	+					+				+				

+: Tissue examined microscopically
A: Autolysis precludes examination

M: Missing tissue
I: Insufficient tissue

X: Lesion present
Blank: Not examined

[illegible]

TABLE B2
Individual Animal Tumor Pathology of Female Rats in the 2-Year Dermal Study
of Benzethonium Chloride: Vehicle Control (continued)

Number of Days on Study	1	4	5	5	5	5	6	6	6	6	6	6	6	6	6	6	6	6	6	6	7	7	7	7
	7	8	1	1	2	5	9	0	1	1	3	4	4	4	4	4	4	6	9	0	1	1	2	2
	6	5	5	9	6	4	5	3	0	2	3	2	2	4	5	8	9	7	6	9	8	9	0	2
Carcass ID Number	2	2	2	2	2	2	2	2	3	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2
	5	7	9	5	8	7	9	6	0	7	6	9	6	7	4	5	8	4	9	9	8	7	9	
	7	5	7	1	1	2	4	1	0	0	3	6	2	9	3	9	3	9	9	1	4	3	5	
Hematopoietic System (continued)																								
Lymph node, mesenteric				+							+	+	+									+		
Spleen	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Thymus	+	+	+	M	+	+	+	+	+	+	+	+	+	+	+	M	+	+	+	+	M	+	+	M
Integumentary System																								
Mammary gland	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Adenoma																	X							
Adenoma, multiple																								
Fibroadenoma									X									X			X	X	X	X
Fibroadenoma, multiple																								
Skin, control	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Basosquamous tumor benign																								X
Skin, site of application-no mass	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Musculoskeletal System																								
Bone	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Nervous System																								
Brain	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Carcinoma, metastatic, pituitary gland																								
Peripheral nerve											+													
Spinal cord											+													
Respiratory System																								
Lung	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Carcinoma, metastatic, thyroid gland																								
Nose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Nares, squamous cell papilloma																	X							
Vomeranosal organ, squamous cell carcinoma																								
Trachea	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Special Senses System																								
Eye							+						+	+										
Harderian gland	+																							
Urinary System																								
Kidney	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Urinary bladder	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Systemic Lesions																								
Multiple organs	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Leukemia mononuclear		X									X	X	X				X		X	X	X	X	X	X

TABLE B2
Individual Animal Tumor Pathology of Female Rats in the 2-Year Dermal Study
of Benzethonium Chloride: Vehicle Control (continued)

[illegible]

TABLE B2

Individual Animal Tumor Pathology of Female Rats in the 2-Year Dermal Study of Benzethonium Chloride: 0.15 mg/kg

Number of Days on Study	3	3	4	5	5	5	5	5	6	6	6	6	6	6	6	6	6	6	7	7	7	7	7	7	7
	6	9	5	1	3	3	5	8	0	1	1	3	3	5	7	8	8	9	1	2	3	3	3	3	3
	6	3	6	9	0	8	1	2	2	2	2	3	8	9	7	0	4	6	0	7	0	0	0	0	0
Carcass ID Number	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3
	3	3	2	6	0	2	0	4	0	3	4	3	2	1	4	4	5	0	0	5	0	0	0	1	1
	7	2	0	0	5	6	2	2	3	8	5	9	1	6	9	1	5	8	1	3	6	7	9	0	1
Alimentary System																									
None																									
Cardiovascular System																									
None																									
Endocrine System																									
None																									
General Body System																									
None																									
Genital System																									
None																									
Hematopoietic System																									
None																									
Integumentary System																									
Skin, control	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Skin, site of application-no mass	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Musculoskeletal System																									
None																									
Nervous System																									
None																									
Respiratory System																									
None																									
Special Senses System																									
None																									
Urinary System																									
None																									
Systemic Lesions																									
Multiple organs	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

[illegible]

TABLE B2

Individual Animal Tumor Pathology of Female Rats in the 2-Year Dermal Study of Benzethonium Chloride: 0.5 mg/kg

Number of Days on Study	2	4	4	5	5	5	5	5	5	5	5	6	6	6	6	6	6	6	6	6	7	7	7	7	7
	9	6	9	3	4	6	7	7	7	8	0	1	3	6	7	7	8	8	9	9	0	0	1	2	2
	7	5	2	3	9	5	8	9	9	1	6	0	2	8	8	9	5	6	4	8	4	8	2	0	5
Carcass ID Number	3	4	4	3	3	4	4	3	4	3	3	3	3	4	3	3	4	3	3	3	4	3	3	4	4
	7	0	0	7	6	1	0	6	0	9	8	7	7	1	7	6	0	6	9	9	1	7	8	0	1
	6	3	2	0	2	1	0	5	8	0	3	8	5	6	2	1	4	8	9	6	0	7	4	9	7
Alimentary System																									
None																									
Cardiovascular System																									
None																									
Endocrine System																									
None																									
General Body System																									
None																									
Genital System																									
None																									
Hematopoietic System																									
None																									
Integumentary System																									
Skin, control	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Trichoepithelioma																									
Subcutaneous tissue, neurofibrosarcoma																									
Skin, site of application-no mass	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Musculoskeletal System																									
None																									
Nervous System																									
None																									
Respiratory System																									
None																									
Special Senses System																									
None																									
Urinary System																									
None																									
Systemic Lesions																									
Multiple organs	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

Individual Animal Tumor Pathology of Female Rats in the 2-Year Dermal Study of Benzethonium Chloride: 0.5 mg/kg
(continued)

[illegible]

TABLE B2
Individual Animal Tumor Pathology of Female Rats in the 2-Year Dermal Study of Benzethonium Chloride: 1.5 mg/kg

[illegible]

TABLE B2

Individual Animal Tumor Pathology of Female Rats in the 2-Year Dermal Study of Benzethonium Chloride: 1.5 mg/kg
(continued)

[illegible]

Individual Animal Tumor Pathology of Female Rats in the 2-Year Dermal Study of Benzethonium Chloride: 1.5 mg/kg
(continued)

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TABLE B2
Individual Animal Tumor Pathology of Female Rats in the 2-Year Dermal Study of Benzethonium Chloride: 1.5 mg/kg
 (continued)

[illegible]

TABLE B3
Statistical Analysis of Primary Neoplasms in Female Rats in the 2-Year Dermal Study of Benzethonium Chloride

	Vehicle Control	0.15 mg/kg	0.5 mg/kg	1.5 mg/kg
Adrenal Medulla: Benign Pheochromocytoma				
Overall rate ^a	3/51 (6%)	— ^e	—	1/53 (2%)
Adjusted rate ^b	12.5%			2.7%
Terminal rate ^c	3/24 (13%)			0/24 (0%)
First incidence (days)	729 (T)			634
Life table test ^d				P=0.310N
Logistic regression test ^d				P=0.325N
Fisher exact test ^d				P=0.294N
Clitoral Gland: Adenoma				
Overall rate	3/50 (6%)	—	—	4/52 (8%)
Adjusted rate	10.4%			16.7%
Terminal rate	2/24 (8%)			4/24 (17%)
First incidence (days)	572			729 (T)
Life table test				P=0.500
Logistic regression test				P=0.529
Fisher exact test				P=0.522
Clitoral Gland: Adenoma or Carcinoma				
Overall rate	3/50 (6%)	—	—	6/52 (12%)
Adjusted rate	10.4%			25.0%
Terminal rate	2/24 (8%)			6/24 (25%)
First incidence (days)	572			729 (T)
Life table test				P=0.237
Logistic regression test				P=0.257
Fisher exact test				P=0.264
Mammary Gland: Adenoma				
Overall rate	3/51 (6%)	—	—	2/53 (4%)
Adjusted rate	10.5%			7.2%
Terminal rate	1/24 (4%)			1/24 (4%)
First incidence (days)	648			658
Life table test				P=0.518N
Logistic regression test				P=0.518N
Fisher exact test				P=0.482N
Mammary Gland: Adenoma or Carcinoma				
Overall rate	3/51 (6%)	—	—	3/53 (6%)
Adjusted rate	10.5%			11.2%
Terminal rate	1/24 (4%)			2/24 (8%)
First incidence (days)	648			658
Life table test				P=0.645
Logistic regression test				P=0.640
Fisher exact test				P=0.642N

TABLE B3

Statistical Analysis of Primary Neoplasms in Female Rats in the 2-Year Dermal Study of Benzethonium Chloride
(continued)

	Vehicle Control	0.15 mg/kg	0.5 mg/kg	1.5 mg/kg
Mammary Gland: Fibroadenoma				
Overall rate	17/51 (33%)	–	–	17/53 (32%)
Adjusted rate	53.6%			56.2%
Terminal rate	10/24 (42%)			12/24 (50%)
First incidence (days)	572			525
Life table test				P=0.545
Logistic regression test				P=0.517
Fisher exact test				P=0.529N
Mammary Gland: Fibroma, Fibroadenoma, or Adenoma				
Overall rate	19/51 (37%)	–	–	19/53 (36%)
Adjusted rate	56.6%			61.1%
Terminal rate	10/24 (42%)			13/24 (54%)
First incidence (days)	572			525
Life table test				P=0.535
Logistic regression test				P=0.514
Fisher exact test				P=0.522N
Mammary Gland: Fibroma, Fibroadenoma, Adenoma, or Carcinoma				
Overall rate	19/51 (37%)	–	–	20/53 (38%)
Adjusted rate	56.6%			64.7%
Terminal rate	10/24 (42%)			14/24 (58%)
First incidence (days)	572			525
Life table test				P=0.458
Logistic regression test				P=0.424
Fisher exact test				P=0.560
Pituitary Gland (Pars Distalis): Adenoma				
Overall rate	28/51 (55%)	–	–	27/53 (51%)
Adjusted rate	73.9%			65.5%
Terminal rate	15/24 (63%)			11/24 (46%)
First incidence (days)	515			486
Life table test				P=0.550N
Logistic regression test				P=0.533N
Fisher exact test				P=0.418N
Pituitary Gland (Pars Distalis): Adenoma or Carcinoma				
Overall rate	30/51 (59%)	–	–	29/53 (55%)
Adjusted rate	77.8%			67.6%
Terminal rate	16/24 (67%)			11/24 (46%)
First incidence (days)	515			486
Life table test				P=0.552N
Logistic regression test				P=0.546N
Fisher exact test				P=0.411N

TABLE B3
Statistical Analysis of Primary Neoplasms in Female Rats in the 2-Year Dermal Study of Benzethonium Chloride
(continued)

	Vehicle Control	0.15 mg/kg	0.5 mg/kg	1.5 mg/kg
Thyroid Gland (C-cell): Adenoma				
Overall rate	11/51 (22%)	—	—	5/53 (9%)
Adjusted rate	35.1%			18.8%
Terminal rate	6/24 (25%)			4/24 (17%)
First incidence (days)	515			613
Life table test				P=0.099N
Logistic regression test				P=0.095N
Fisher exact test				P=0.074N
Uterus: Stromal Polyp				
Overall rate	3/51 (6%)	—	—	7/53 (13%)
Adjusted rate	10.2%			22.0%
Terminal rate	2/24 (8%)			4/24 (17%)
First incidence (days)	485			533
Life table test				P=0.166
Logistic regression test				P=0.181
Fisher exact test				P=0.176
All Organs: Mononuclear Cell Leukemia				
Overall rate	18/51 (35%)	—	—	18/53 (34%)
Adjusted rate	47.8%			53.8%
Terminal rate	6/24 (25%)			10/24 (42%)
First incidence (days)	485			303
Life table test				P=0.516
Logistic regression test				P=0.574N
Fisher exact test				P=0.525N
All Organs: Benign Neoplasms				
Overall rate	39/51 (76%)	—	—	42/53 (79%)
Adjusted rate	88.4%			93.0%
Terminal rate	19/24 (79%)			21/24 (88%)
First incidence (days)	485			486
Life table test				P=0.323
Logistic regression test				P=0.354
Fisher exact test				P=0.458
All Organs: Malignant Neoplasms				
Overall rate	23/51 (45%)	—	—	21/53 (40%)
Adjusted rate	59.2%			59.5%
Terminal rate	9/24 (38%)			11/24 (46%)
First incidence (days)	485			303
Life table test				P=0.494N
Logistic regression test				P=0.413N
Fisher exact test				P=0.357N

TABLE B3

Statistical Analysis of Primary Neoplasms in Female Rats in the 2-Year Dermal Study of Benzethonium Chloride
(continued)

	Vehicle Control	0.15 mg/kg	0.5 mg/kg	1.5 mg/kg
All Organs: Benign or Malignant Neoplasms^a				
Overall rate	43/51 (84%)	–	–	49/53 (92%)
Adjusted rate	91.4%			98.0%
Terminal rate	20/24 (83%)			23/24 (96%)
First incidence (days)	485			303
Life table test				P=0.191
Logistic regression test				P=0.105
Fisher exact test				P=0.161

(T) Terminal sacrifice

^a Number of neoplasm-bearing animals/number of animals examined. Denominator is number of animals examined microscopically for adrenal gland, bone marrow, brain, clitoral gland, heart, kidney, larynx, liver, lung, nose, ovary, pancreas, parathyroid gland, pituitary gland, salivary gland, spleen, thyroid gland, and urinary bladder; for other tissues, denominator is number of animals necropsied.

^b Kaplan-Meier estimated neoplasm incidence at the end of the study after adjustment for intercurrent mortality

^c Observed incidence at terminal kill

^d Beneath the dosed group incidence are the P values corresponding to pairwise comparisons between the controls and that dosed group. The life table test regards neoplasms in animals dying prior to terminal kill as being (directly or indirectly) the cause of death. The logistic regression test regards these lesions as nonfatal. The Fisher exact test compares directly the overall incidence rates. For all tests, a lower incidence in a dose group is indicated by N.

^e Organ was not examined at this dose level

TABLE B4

Summary of the Incidence of Nonneoplastic Lesions in Female Rats in the 2-Year Dermal Study of Benzethonium Chloride^a

	Vehicle Control	0.15 mg/kg	0.5 mg/kg	1.5 mg/kg
Disposition Summary				
Animals initially in study	60	60	60	60
15-Month interim evaluation	9	7	9	7
Early deaths				
Moribund	13	11	13	13
Natural deaths	14	9	12	16
Survivors				
Terminal sacrifice	24	33	26	24
Animals examined microscopically	60	60	60	60
15-Month Interim Evaluation				
Alimentary System				
Liver	(9)			(7)
Hepatodiaphragmatic nodule	2 (22%)			3 (43%)
Hepatodiaphragmatic nodule, multiple	1 (11%)			
Inflammation, chronic active	2 (22%)			2 (29%)
Mesentery	(1)			(1)
Fat, inflammation, chronic active				1 (100%)
Pancreas	(9)			(7)
Acinus, atrophy				2 (29%)
Cardiovascular System				
Heart	(9)			(7)
Degeneration, chronic	1 (11%)			3 (43%)
Endocrine System				
Pituitary gland	(9)			(7)
Craniopharyngeal duct, pars distalis, cyst				1 (14%)
Pars distalis, cyst	4 (44%)			1 (14%)
Pars distalis, hyperplasia	3 (33%)			3 (43%)
Thyroid gland	(9)			(7)
Bilateral, ultimobranchial cyst				1 (14%)
C-cell, hyperplasia	1 (11%)			
Genital System				
Clitoral gland	(9)			(7)
Inflammation, chronic active	1 (11%)			
Duct, cyst	1 (11%)			
Ovary	(9)			(7)
Periovarian tissue, cyst	3 (33%)			3 (43%)
Uterus	(9)			(7)
Endometrium, hyperplasia, cystic, glandular				1 (14%)
Hematopoietic System				
Thymus	(8)			(6)
Angiectasis				1 (17%)
Depletion lymphoid	8 (100%)			6 (100%)

TABLE B4

Summary of the Incidence of Nonneoplastic Lesions in Female Rats in the 2-Year Dermal Study of Benzethonium Chloride (continued)

	Vehicle Control	0.15 mg/kg	0.5 mg/kg	1.5 mg/kg
15-Month Interim Evaluation (continued)				
Integumentary System				
Mammary gland	(9)			(7)
Hyperplasia, cystic	8 (89%)			5 (71%)
Skin, control	(9)	(7)	(9)	(7)
Skin, site of application-no mass	(9)	(7)	(9)	(7)
Epithelial hyperplasia		1 (14%)	2 (22%)	6 (86%)
Erosion, focal				1 (14%)
Ulcer		1 (14%)	1 (11%)	4 (57%)
Sebaceous gland, hyperplasia		1 (14%)	1 (11%)	6 (86%)
Respiratory System				
Nose	(9)			(7)
Submucosa, inflammation, chronic	1 (11%)			
Urinary System				
Kidney	(9)			(7)
Mineralization	7 (78%)			7 (100%)
Nephropathy, chronic	3 (33%)			5 (71%)
Systems Examined With No Lesions Observed				
General Body System				
Musculoskeletal System				
Nervous System				
Special Senses System				
2-Year Study				
Alimentary System				
Intestine small, duodenum	(2)			
Ulcer	2 (100%)			
Liver	(51)			(53)
Angiectasis	2 (4%)			
Basophilic focus	26 (51%)			33 (62%)
Clear cell focus	2 (4%)			7 (13%)
Eosinophilic focus	13 (25%)			13 (25%)
Hepatodiaphragmatic nodule	7 (14%)			15 (28%)
Inflammation, granulomatous	8 (16%)			11 (21%)
Bile duct, hyperplasia	11 (22%)			13 (25%)
Hepatocyte, degeneration, cystic				2 (4%)
Hepatocyte, hypertrophy, focal				1 (2%)
Hepatocyte, necrosis	1 (2%)			
Hepatocyte, vacuolization cytoplasmic	7 (14%)			8 (15%)
Mesentery	(3)			(3)
Fat, inflammation, chronic active	3 (100%)			3 (100%)
Pancreas	(51)			(53)
Inflammation, chronic active				1 (2%)
Acinus, atrophy	15 (29%)			15 (28%)

TABLE B4
Summary of the Incidence of Nonneoplastic Lesions in Female Rats in the 2-Year Dermal Study
of Benzethonium Chloride (continued)

	Vehicle Control	0.15 mg/kg	0.5 mg/kg	1.5 mg/kg
2-Year Study (continued)				
Alimentary System (continued)				
Stomach, forestomach	(6)			(8)
Acanthosis				3 (38%)
Inflammation, chronic active				1 (13%)
Mineralization				1 (13%)
Ulcer	6 (100%)			5 (63%)
Stomach, glandular	(1)			
Necrosis	1 (100%)			
Tongue				(1)
Cyst				1 (100%)
Cardiovascular System				
Heart	(51)			(53)
Degeneration, chronic	28 (55%)			30 (57%)
Atrium, thrombosis	3 (6%)			3 (6%)
Endocrine System				
Adrenal cortex	(19)			(21)
Degeneration, cystic	1 (5%)			1 (5%)
Hyperplasia	7 (37%)			12 (57%)
Adrenal cortex (continued)				
Hypertrophy				1 (5%)
Necrosis	2 (11%)			2 (10%)
Vacuolization cytoplasmic	8 (42%)			9 (43%)
Adrenal medulla	(10)			(2)
Hyperplasia	6 (60%)			1 (50%)
Parathyroid gland	(47)			(47)
Hyperplasia	32 (68%)			32 (68%)
Pituitary gland	(51)			(53)
Necrosis	1 (2%)			
Pars distalis, cyst	7 (14%)			11 (21%)
Pars distalis, hyperplasia	14 (27%)			17 (32%)
Thyroid gland	(51)			(53)
C-cell, hyperplasia	23 (45%)			17 (32%)
Follicular cell, hyperplasia				2 (4%)
General Body System				
None				
Genital System				
Clitoral gland	(50)			(52)
Hyperplasia	5 (10%)			4 (8%)
Inflammation, chronic active	1 (2%)			1 (2%)
Duct, ectasia	1 (2%)			2 (4%)
Ovary	(51)			(53)
Follicle, cyst	4 (8%)			7 (13%)
Periovarian tissue, cyst	5 (10%)			10 (19%)

TABLE B4
Summary of the Incidence of Nonneoplastic Lesions in Female Rats in the 2-Year Dermal Study
of Benzethonium Chloride (continued)

	Vehicle Control	0.15 mg/kg	0.5 mg/kg	1.5 mg/kg
2-Year Study (continued)				
Genital System (continued)				
Uterus	(51)			(53)
Endometrium, hyperplasia, cystic, glandular	3 (6%)			2 (4%)
Vagina	(1)			
Lumen, hemorrhage	1 (100%)			
Hematopoietic System				
Blood	(2)			
Erythrocyte, atypia cellular	1 (50%)			
Bone marrow	(51)			(52)
Femoral, myelofibrosis	1 (2%)			
Lymph node, mandibular	(7)			(3)
Thrombosis	1 (14%)			
Lymph node, mesenteric	(5)			(3)
Angiectasis				2 (67%)
Spleen	(51)			(53)
Fibrosis	1 (2%)			2 (4%)
Hematopoietic cell proliferation	1 (2%)			2 (4%)
Thymus	(46)			(50)
Cyst				1 (2%)
Depletion lymphoid				3 (6%)
Integumentary System				
Mammary gland	(51)			(53)
Hyperplasia, cystic	50 (98%)			51 (96%)
Skin, control	(51)	(53)	(51)	(53)
Epithelial hyperplasia		3 (6%)	2 (4%)	
Sebaceous gland, hyperplasia		1 (2%)	1 (2%)	
Skin, site of application-no mass	(51)	(53)	(51)	(53)
Epithelial hyperplasia	2 (4%)	2 (4%)	6 (12%)	32 (60%)
Ulcer		1 (2%)	3 (6%)	19 (36%)
Sebaceous gland, hyperplasia	1 (2%)	2 (4%)	6 (12%)	30 (57%)
Musculoskeletal System				
Bone	(51)			(53)
Femur, hyperostosis	2 (4%)			2 (4%)
Femur, osteopetrosis				1 (2%)
Nervous System				
Brain	(51)			(53)
Compression	21 (41%)			17 (32%)
Hemorrhage	1 (2%)			
Hydrocephalus	8 (16%)			6 (11%)

TABLE B4

Summary of the Incidence of Nonneoplastic Lesions in Female Rats in the 2-Year Dermal Study of Benzethonium Chloride (continued)

	Vehicle Control	0.15 mg/kg	0.5 mg/kg	1.5 mg/kg
2-Year Study (continued)				
Respiratory System				
Lung	(51)			(53)
Inflammation, chronic active	2 (4%)			2 (4%)
Alveolar epithelium, hyperplasia				1 (2%)
Perivascular, inflammation, chronic	1 (2%)			
Nose	(51)			(53)
Mucosa, inflammation, chronic active	4 (8%)			5 (9%)
Sinus, foreign Body	3 (6%)			5 (9%)
Special Senses System				
Eye	(6)			(4)
Phthisis bulbi	1 (17%)			
Lens, cataract	5 (83%)			4 (100%)
Retina, atrophy	5 (83%)			4 (100%)
Harderian gland	(1)			(1)
Inflammation, chronic active	1 (100%)			1 (100%)
Urinary System				
Kidney	(51)			(53)
Cyst	1 (2%)			
Mineralization	1 (2%)			2 (4%)
Nephropathy, chronic	45 (88%)			48 (91%)
Urinary bladder	(51)			(53)
Transitional epithelium, hyperplasia				1 (2%)

^a Number of animals examined microscopically at the site and the number of animals with lesion

APPENDIX C

SUMMARY OF LESIONS IN MALE MICE IN THE 2-YEAR DERMAL STUDY OF BENZETHONIUM CHLORIDE

TABLE C1	Summary of the Incidence of Neoplasms in Male Mice in the 2-Year Dermal Study of Benzethonium Chloride	C-3
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TABLE C1

Summary of the Incidence of Neoplasms in Male Mice in the 2-Year Dermal Study of Benzethonium Chloride^a

	Vehicle Control	0.15 mg/kg	0.5 mg/kg	1.5 mg/kg
Disposition Summary				
Animals initially in study	60	60	60	60
<i>15-Month interim evaluation</i>	10	9	9	10
Early deaths				
Moribund	3	8	4	9
Natural deaths	4	4	4	2
Survivors				
Terminal sacrifice	43	38	42	39
Missexed		1	1	
Animals examined microscopically	60	59	59	60
15-Month Interim Evaluation				
Alimentary System				
Liver	(10)			(10)
Hepatocellular carcinoma	1 (10%)			2 (20%)
Hepatocellular carcinoma, multiple	1 (10%)			
Hepatocellular adenoma	4 (40%)			4 (40%)
Integumentary System				
Skin, control	(10)	(9)	(9)	(10)
Skin, site of application	(10)	(9)	(9)	(10)
Special Senses System				
Harderian gland				(1)
Adenoma				1 (100%)
Systems Examined With No Neoplasms Observed				
Cardiovascular System				
Endocrine System				
General Body System				
Genital System				
Hematopoietic System				
Musculoskeletal System				
Nervous System				
Respiratory System				
Urinary System				

TABLE C1

Summary of the Incidence of Neoplasms in Male Mice in the 2-Year Dermal Study of Benzethonium Chloride (continued)

	Vehicle Control	0.15 mg/kg	0.5 mg/kg	1.5 mg/kg
2-Year Study				
Alimentary System				
Intestine large, cecum	(50)			(50)
Leiomyosarcoma				1 (2%)
Intestine small, duodenum	(50)			(50)
Adenoma				1 (2%)
Intestine small, jejunum	(50)			(50)
Liver	(50)			(50)
Hemangioma				1 (2%)
Hemangiosarcoma	2 (4%)			
Hemangiosarcoma, multiple	1 (2%)			2 (4%)
Hemangiosarcoma, metastatic, skin	1 (2%)			
Hemangiosarcoma, metastatic, spleen				1 (2%)
Hepatoblastoma				1 (2%)
Hepatocellular carcinoma	9 (18%)			12 (24%)
Hepatocellular carcinoma, multiple	1 (2%)			2 (4%)
Hepatocellular adenoma	11 (22%)			12 (24%)
Hepatocellular adenoma, multiple	13 (26%)			13 (26%)
Histiocytic sarcoma				1 (2%)
Mesentery	(4)			(4)
Hemangioma	1 (25%)			1 (25%)
Histiocytic sarcoma				1 (25%)
Stomach, glandular	(50)			(50)
Carcinoid tumor malignant				1 (2%)
Cardiovascular System				
Heart	(50)			(50)
Hemangiosarcoma, metastatic, liver	1 (2%)			1 (2%)
Endocrine System				
Adrenal cortex	(50)			(50)
Histiocytic sarcoma				1 (2%)
Adrenal medulla	(50)			(50)
Pheochromocytoma benign	1 (2%)			
Islets, pancreatic	(49)			(50)
Adenoma	2 (4%)			1 (2%)
Pituitary gland	(48)			(48)
Histiocytic sarcoma				1 (2%)
Thyroid gland	(50)			(50)
Hemangiosarcoma, metastatic, spleen				1 (2%)
Follicular cell, adenoma				1 (2%)
Follicular cell, carcinoma	1 (2%)			1 (2%)
General Body System				
None				
Genital System				
Epididymis	(50)			(49)
Histiocytic sarcoma				1 (2%)
Testes	(50)			(50)
Interstitial cell, adenoma	1 (2%)			1 (2%)

TABLE C1

Summary of the Incidence of Neoplasms in Male Mice in the 2-Year Dermal Study of Benzethonium Chloride (continued)

	Vehicle Control	0.15 mg/kg	0.5 mg/kg	1.5 mg/kg
2-Year Study (continued)				
Hematopoietic System				
Bone marrow	(50)			(50)
Hemangiosarcoma, metastatic, liver	1 (2%)			
Hemangiosarcoma, metastatic, skin	1 (2%)			
Hemangiosarcoma, metastatic, spleen				1 (2%)
Histiocytic sarcoma				1 (2%)
Lymph node	(3)			(2)
Lymph node, mandibular	(49)			(48)
Lymph node, mesenteric	(48)			(47)
Spleen	(50)			(50)
Hemangiosarcoma				3 (6%)
Hemangiosarcoma, metastatic, liver	1 (2%)			
Histiocytic sarcoma				1 (2%)
Thymus	(31)			(39)
Integumentary System				
Skin, control	(50)	(50)	(50)	(50)
Melanoma benign				1 (2%)
Subcutaneous tissue, hemangioma	1 (2%)			
Subcutaneous tissue, hemangiosarcoma	1 (2%)			1 (2%)
Subcutaneous tissue, hemangiosarcoma, metastatic, spleen				1 (2%)
Skin, site of application-no mass	(50)	(50)	(50)	(50)
Musculoskeletal System				
None				
Nervous System				
Brain	(50)			(50)
Histiocytic sarcoma				1 (2%)
Respiratory System				
Lung	(50)			(50)
Alveolar/bronchiolar adenoma	11 (22%)			7 (14%)
Alveolar/bronchiolar adenoma, multiple	2 (4%)			1 (2%)
Alveolar/bronchiolar carcinoma	1 (2%)			
Alveolar/bronchiolar carcinoma, multiple				2 (4%)
Carcinoma, metastatic, harderian gland	1 (2%)			
Carcinoma, metastatic, thyroid gland				1 (2%)
Hepatocellular carcinoma, metastatic, liver	2 (4%)			5 (10%)
Histiocytic sarcoma				1 (2%)
Nose	(50)			(50)
Glands, carcinoma				1 (2%)

TABLE C1

Summary of the Incidence of Neoplasms in Male Mice in the 2-Year Dermal Study of Benzethonium Chloride (continued)

	Vehicle Control	0.15 mg/kg	0.5 mg/kg	1.5 mg/kg
2-Year Study (continued)				
Special Senses System				
Ear	(1)			(2)
Fibrosarcoma	1 (100%)			1 (50%)
Harderian gland	(43)			(37)
Adenoma	2 (5%)			2 (5%)
Carcinoma	1 (2%)			
Urinary System				
Kidney	(50)			(50)
Histiocytic sarcoma				1 (2%)
Systemic Lesions				
Multiple organs ^b	(50)	(50)	(50)	(50)
Histiocytic sarcoma				1 (2%)
Lymphoma malignant lymphocytic	1 (2%)			
Lymphoma malignant mixed	1 (2%)			3 (6%)
Lymphoma malignant undifferentiated cell	1 (2%)			

TABLE C1
Summary of the Incidence of Neoplasms in Male Mice in the 2-Year Dermal Study of Benzethonium Chloride (continued)

	Vehicle Control	0.15 mg/kg	0.5 mg/kg	1.5 mg/kg
Neoplasm Summary				
Total animals with primary neoplasms ^c				
15-Month interim evaluation	5			4
2-Year study	43			40
Total primary neoplasms				
15-Month interim evaluation	6			7
2-Year study	66			74
Total animals with benign neoplasms				
15-Month interim evaluation	4			4
2-Year study	35			30
Total benign neoplasms				
15-Month interim evaluation	4			4
2-Year study	45			42
Total animals with malignant neoplasms				
15-Month interim evaluation	2			2
2-Year study	16			22
Total malignant neoplasms				
15-Month interim evaluation	2			2
2-Year study	21			32
Total animals with metastatic neoplasms				
2-Year study	4			8
Total metastatic neoplasms				
2-Year study	8			11

^a Number of animals examined microscopically at the site and the number of animals with neoplasm

^b Number of animals with any tissue examined microscopically

^c Primary neoplasms: all neoplasms except metastatic neoplasms

[illegible]

X: Lesion present
Blank: Not examined

TABLE C2
Individual Animal Tumor Pathology of Male Mice in the 2-Year Dermal Study
of Benzethonium Chloride: Vehicle Control (continued)

[illegible]

TABLE C2

[illegible]

[illegible]

TABLE C2
Individual Animal Tumor Pathology of Male Mice in the 2-Year Dermal Study
of Benzethonium Chloride: Vehicle Control (continued)

	5	6	6	6	6	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7
Number of Days on Study	7	0	1	3	5	1	1	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3
	7	3	3	7	5	7	9	2	2	2	2	2	2	2	2	2	2	2	3	3	3	3	3	3
Carcass ID Number	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	5	2	1	3	0	1	4	0	0	0	0	0	0	0	0	1	1	1	1	1	2	2	2	2
	7	3	9	9	9	2	5	1	2	3	4	5	6	7	8	1	3	4	6	7	1	2	4	5
Systemic Lesions																								
Multiple organs	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Lymphoma malignant lymphocytic																								
Lymphoma malignant mixed																								
Lymphoma malignant undifferentiated cell type																								

TABLE C2

	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7
Number of Days on Study	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3
	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3
Carcass ID Number	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	2	3	3	3	3	3	3	3	4	4	4	4	4	4	4	4	5	5	5	5	5	5	5	5	6
	8	0	1	2	4	5	6	7	0	1	2	3	6	7	8	9	0	2	3	4	5	6	8	9	0
Total Tissues/Tumors																									
Systemic Lesions																									
Multiple organs	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	50
Lymphoma malignant lymphocytic																									1
Lymphoma malignant mixed																									1
Lymphoma malignant undifferentiated cell type																						X			1

TABLE C2
Individual Animal Tumor Pathology of Male Mice in the 2-Year Dermal Study of Benzethonium Chloride: 0.15 mg/kg

[illegible]

Individual Animal Tumor Pathology of Male Mice in the 2-Year Dermal Study of Benzethonium Chloride: 0.15 mg/kg
(continued)

[illegible]

Individual Animal Tumor Pathology of Male Mice in the 2-Year Dermal Study of Benzethonium Chloride: 0.5 mg/kg

Board Draft

Individual Animal Tumor Pathology of Male Mice in the 2-Year Dermal Study of Benzethonium Chloride: 0.5 mg/kg
(continued)

[illegible]

Individual Animal Tumor Pathology of Male Mice in the 2-Year Dermal Study of Benzethonium Chloride: 1.5 mg/kg

Board Draft

TABLE C2
Individual Animal Tumor Pathology of Male Mice in the 2-Year Dermal Study of Benzethonium Chloride: 1.5 mg/kg
 (continued)

[illegible]

TABLE C2
Individual Animal Tumor Pathology of Male Mice in the 2-Year Dermal Study of Benzethonium Chloride: 1.5 mg/kg
(continued)

[illegible]

TABLE C2
Individual Animal Tumor Pathology of Male Mice in the 2-Year Dermal Study of Benzethonium Chloride: 1.5 mg/kg
 (continued)

[illegible]

Individual Animal Tumor Pathology of Male Mice in the 2-Year Dermal Study of Benzethonium Chloride: 1.5 mg/kg
(continued)

Board Draft

TABLE C2
Individual Animal Tumor Pathology of Male Mice in the 2-Year Dermal Study of Benzethonium Chloride: 1.5 mg/kg
(continued)

[illegible]

TABLE C3
Statistical Analysis of Primary Neoplasms in Male Mice in the 2-Year Dermal Study of Benzethonium Chloride

	Vehicle Control	0.15 mg/kg	0.5 mg/kg	1.5 mg/kg
Harderian Gland: Adenoma or Carcinoma				
Overall rate ^a	3/50 (6%)	— ^c	—	2/50 (4%)
Adjusted rate	6.7%			4.9%
Terminal rate	2/43 (5%)			1/39 (3%)
First incidence (days)	655			712
Life table test				P=0.531N
Logistic regression test ^d				P=0.495N
Fisher exact test ^e				P=0.500N
Liver: Hemangiosarcoma				
Overall rate	3/50 (6%)	—	—	2/50 (4%)
Adjusted rate	6.4%			4.9%
Terminal rate	0/43 (0%)			1/39 (3%)
First incidence (days)	577			719
Life table test				P=0.531N
Logistic regression test				P=0.307N
Fisher exact test				P=0.500N
Liver: Hepatocellular Adenoma				
Overall rate	24/50 (48%)	—	—	25/50 (50%)
Adjusted rate	53.2%			55.2%
Terminal rate	22/43 (51%)			19/39 (49%)
First incidence (days)	603			584
Life table test				P=0.347
Logistic regression test				P=0.505
Fisher exact test				P=0.500
Liver: Hepatocellular Carcinoma				
Overall rate	10/50 (20%)	—	—	14/50 (28%)
Adjusted rate	21.6%			32.4%
Terminal rate	7/43 (16%)			10/39 (26%)
First incidence (days)	603			684
Life table test				P=0.189
Logistic regression test				P=0.363
Fisher exact test				P=0.241
Liver: Hepatocellular Adenoma or Carcinoma				
Overall rate	29/50 (58%)	—	—	33/50 (66%)
Adjusted rate	63.0%			70.1%
Terminal rate	26/43 (60%)			25/39 (64%)
First incidence (days)	603			584
Life table test				P=0.153
Logistic regression test				P=0.331
Fisher exact test				P=0.268

TABLE C3

Statistical Analysis of Primary Neoplasms in Male Mice in the 2-Year Dermal Study of Benzethonium Chloride
(continued)

	Vehicle Control	0.15 mg/kg	0.5 mg/kg	1.5 mg/kg
Liver: Hepatoblastoma or Hepatocellular Carcinoma				
Overall rate	10/50 (20%)	—	—	14/50 (28%)
Adjusted rate	21.6%			32.4%
Terminal rate	7/43 (16%)			10/39 (26%)
First incidence (days)	603			684
Life table test				P=0.189
Logistic regression test				P=0.363
Fisher exact test				P=0.241
Liver: Hepatoblastoma, Hepatocellular Adenoma, or Carcinoma				
Overall rate	29/50 (58%)	—	—	33/50 (66%)
Adjusted rate	63.0%			70.1%
Terminal rate	26/43 (60%)			25/39 (64%)
First incidence (days)	603			584
Life table test				P=0.153
Logistic regression test				P=0.331
Fisher exact test				P=0.268
Lung: Alveolar/bronchiolar Adenoma				
Overall rate	13/50 (26%)	—	—	8/50 (16%)
Adjusted rate	29.4%			19.3%
Terminal rate	12/43 (28%)			6/39 (15%)
First incidence (days)	637			687
Life table test				P=0.228N
Logistic regression test				P=0.181N
Fisher exact test				P=0.163N
Lung: Alveolar/bronchiolar Adenoma or Carcinoma				
Overall rate	14/50 (28%)	—	—	10/50 (20%)
Adjusted rate	31.7%			24.2%
Terminal rate	13/43 (30%)			8/39 (21%)
First incidence (days)	637			687
Life table test				P=0.327N
Logistic regression test				P=0.274N
Fisher exact test				P=0.241N
Spleen: Hemangiosarcoma				
Overall rate	0/50 (0%)	—	—	3/50 (6%)
Adjusted rate	0.0%			7.2%
Terminal rate	0/43 (0%)			2/39 (5%)
First incidence (days)	—			687
Life table test				P=0.112
Logistic regression test				P=0.162
Fisher exact test				P=0.121

TABLE C3

Statistical Analysis of Primary Neoplasms in Male Mice in the 2-Year Dermal Study of Benzethonium Chloride
(continued)

	Vehicle Control	0.15 mg/kg	0.5 mg/kg	1.5 mg/kg
All Organs: Hemangiosarcoma				
Overall rate	4/50 (8%)	—	—	6/50 (12%)
Adjusted rate	8.5%	—	—	14.4%
Terminal rate	1/43 (2%)	—	—	4/39 (10%)
First incidence (days)	577	—	—	687
Life table test				P=0.330
Logistic regression test				P=0.382
Fisher exact test				P=0.370
All Organs: Hemangioma or Hemangiosarcoma				
Overall rate	6/50 (12%)	—	—	8/50 (16%)
Adjusted rate	12.9%	—	—	18.6%
Terminal rate	3/43 (7%)	—	—	5/39 (13%)
First incidence (days)	577	—	—	684
Life table test				P=0.340
Logistic regression test				P=0.396
Fisher exact test				P=0.387
All Organs: Malignant Lymphoma (Lymphocytic, Mixed, or Undifferentiated Cell Type)				
Overall rate	3/50 (6%)	—	—	3/50 (6%)
Adjusted rate	7.0%	—	—	6.9%
Terminal rate	3/43 (7%)	—	—	1/39 (3%)
First incidence (days)	729 (T)	—	—	659
Life table test				P=0.628
Logistic regression test				P=0.662N
Fisher exact test				P=0.661N
All Organs: Malignant Lymphoma or Histiocytic Sarcoma				
Overall rate	3/50 (6%)	—	—	4/50 (8%)
Adjusted rate	7.0%	—	—	8.8%
Terminal rate	3/43 (7%)	—	—	1/39 (3%)
First incidence (days)	729 (T)	—	—	612
Life table test				P=0.468
Logistic regression test				P=0.520
Fisher exact test				P=0.500
All Organs: Benign Neoplasms				
Overall rate	37/50 (74%)	—	—	31/50 (62%)
Adjusted rate	77.1%	—	—	65.8%
Terminal rate	32/43 (74%)	—	—	23/39 (59%)
First incidence (days)	603	—	—	584
Life table test				P=0.349N
Logistic regression test				P=0.149N
Fisher exact test				P=0.142N

TABLE C3
Statistical Analysis of Primary Neoplasms in Male Mice in the 2-Year Dermal Study of Benzethonium Chloride
(continued)

	Vehicle Control	0.15 mg/kg	0.5 mg/kg	1.5 mg/kg
All Organs: Malignant Neoplasms				
Overall rate	18/50 (36%)	–	–	24/50 (48%)
Adjusted rate	38.1%			49.9%
Terminal rate	14/43 (33%)			15/39 (38%)
First incidence (days)	577			584
Life table test				P=0.121
Logistic regression test				P=0.164
Fisher exact test				P=0.156
All Organs: Benign or Malignant Neoplasms				
Overall rate	44/50 (88%)	–	–	41/50 (82%)
Adjusted rate	89.8%			83.7%
Terminal rate	38/43 (88%)			31/39 (79%)
First incidence (days)	577			584
Life table test				P=0.533
Logistic regression test				P=0.300N
Fisher exact test				P=0.288N

(T) Terminal sacrifice

- ^a Number of neoplasm-bearing animals/number of animals examined. Denominator is number of animals examined microscopically for adrenal gland, bone marrow, brain, epididymis, gallbladder, heart, kidney, larynx, liver, lung, nose, pancreas, parathyroid gland, pituitary gland, preputial gland, prostate gland, salivary gland, spleen, testes, thyroid gland, and urinary bladder; for other tissues, denominator is number of animals necropsied.
- ^b Kaplan-Meier estimated neoplasm incidence at the end of the study after adjustment for intercurrent mortality
- ^c Observed incidence at terminal kill
- ^d Beneath the dosed group incidence are the P values corresponding to pairwise comparisons between the controls and that dosed group. The life table test regards neoplasms in animals dying prior to terminal kill as being (directly or indirectly) the cause of death. The logistic regression test regards these lesions as nonfatal. The Fisher exact test compares directly the overall incidence rates. For all tests, a lower incidence in a dose group is indicated by N.
- ^e Organ was not examined at this dose level
- ^f Not applicable; no neoplasms in animal group

TABLE C4
Summary of the Incidence of Nonneoplastic Lesions in Male Mice in the 2-Year Dermal Study of Benzethonium Chloride^a

	Vehicle Control	0.15 mg/kg	0.5 mg/kg	1.5 mg/kg
Disposition Summary				
Animals initially in study	60	60	60	60
<i>15-Month interim evaluation</i>	10	9	9	10
Early deaths				
Moribund	3	8	4	9
Natural deaths	4	4	4	2
Survivors				
Terminal sacrifice	43	38	42	39
Missexed		1	1	
Animals examined microscopically	60	59	59	60
15-Month Interim Evaluation				
Alimentary System				
Liver	(10)			(10)
Clear cell focus	2 (20%)			1 (10%)
Fatty change, focal				1 (10%)
Mesentery	(1)			(1)
Fat, necrosis	1 (100%)			1 (100%)
Pancreas	(10)			(10)
Atrophy	1 (10%)			
Cytoplasmic alteration	1 (10%)			
Salivary glands	(10)			(10)
Atrophy	1 (10%)			
Endocrine System				
Adrenal cortex	(10)			(10)
Hyperplasia				1 (10%)
Islets, pancreatic	(10)			(10)
Hyperplasia	1 (10%)			1 (10%)
Pituitary gland	(10)			(10)
Pars intermedia, hyperplasia	1 (10%)			
Genital System				
Preputial gland	(1)			(1)
Duct, ectasia	1 (100%)			1 (100%)
Hematopoietic System				
Spleen	(10)			(10)
Hematopoietic cell proliferation	1 (10%)			2 (20%)
Hyperplasia, lymphoid	1 (10%)			
Thymus	(10)			(10)
Hyperplasia, lymphoid	1 (10%)			
Integumentary System				
Skin, control	(10)	(9)	(9)	(10)
Skin, site of application-no mass	(10)	(9)	(9)	(10)
Epithelial hyperplasia			2 (22%)	10 (100%)

TABLE C4

Summary of the Incidence of Nonneoplastic Lesions in Male Mice in the 2-Year Dermal Study of Benzethonium Chloride (continued)

	Vehicle Control	0.15 mg/kg	0.5 mg/kg	1.5 mg/kg
15-Month Interim Evaluation (continued)				
Respiratory System				
Lung	(10)			(10)
Alveolar epithelium, hyperplasia	1 (10%)			
Urinary System				
Kidney	(10)			(10)
Nephropathy	10 (100%)			9 (90%)
Systems Examined With No Lesions Observed				
Cardiovascular System				
General Body System				
Musculoskeletal System				
Nervous System				
Special Senses System				
2-Year Study				
Alimentary System				
Intestine small, duodenum	(50)			(50)
Erosion	1 (2%)			
Intestine small, jejunum	(50)			(50)
Hyperplasia, lymphoid	1 (2%)			1 (2%)
Inflammation, chronic active				1 (2%)
Liver	(50)			(50)
Basophilic focus	2 (4%)			5 (10%)
Clear cell focus	11 (22%)			11 (22%)
Eosinophilic focus	14 (28%)			16 (32%)
Hematopoietic cell proliferation	2 (4%)			
Infarct	1 (2%)			
Mixed cell focus	5 (10%)			4 (8%)
Necrosis	1 (2%)			2 (4%)
Mesentery	(4)			(4)
Inflammation, chronic active				1 (25%)
Fat, necrosis	3 (75%)			
Vein, thrombosis				1 (25%)
Pancreas	(50)			(50)
Atrophy	2 (4%)			4 (8%)
Atypia cellular	4 (8%)			2 (4%)
Concretion				1 (2%)
Necrosis				1 (2%)
Duct, cyst				1 (2%)
Stomach, forestomach	(50)			(50)
Erosion				2 (4%)
Hyperplasia	1 (2%)			4 (8%)
Stomach, glandular	(50)			(50)
Erosion	1 (2%)			
Tooth	(1)			(2)
Inflammation, chronic active				1 (50%)
Inflammation, suppurative	1 (100%)			1 (50%)

TABLE C4

Summary of the Incidence of Nonneoplastic Lesions in Male Mice in the 2-Year Dermal Study
of Benzethonium Chloride (continued)

	Vehicle Control	0.15 mg/kg	0.5 mg/kg	1.5 mg/kg
2-Year Study (continued)				
Cardiovascular System				
Heart	(50)			(50)
Inflammation, chronic active	2 (4%)			2 (4%)
Artery, inflammation, chronic active	1 (2%)			
Valve, inflammation, chronic active				1 (2%)
Endocrine System				
Adrenal cortex	(50)			(50)
Accessory adrenal cortical nodule				1 (2%)
Hyperplasia	40 (80%)			32 (64%)
Capsule, hyperplasia, adenomatous	5 (10%)			9 (18%)
Islets, pancreatic	(49)			(50)
Hyperplasia	21 (43%)			18 (36%)
Parathyroid gland	(47)			(45)
Cyst	1 (2%)			
Pituitary gland	(48)			(48)
Cyst	2 (4%)			2 (4%)
Pars distalis, hyperplasia				2 (4%)
Pars intermedia, hyperplasia	2 (4%)			1 (2%)
Thyroid gland	(50)			(50)
Inflammation				1 (2%)
Ultimobranchial cyst	1 (2%)			
Follicle, cyst	3 (6%)			1 (2%)
Follicular cell, hyperplasia	10 (20%)			7 (14%)
General Body System				
Tissue NOS				(1)
Hemorrhage				1 (100%)
Genital System				
Epididymis	(50)			(49)
Inflammation	3 (6%)			5 (10%)
Preputial gland	(18)			(17)
Inflammation, chronic active	6 (33%)			3 (18%)
Duct, ectasia	18 (100%)			16 (94%)
Prostate	(49)			(50)
Inflammation, suppurative	1 (2%)			
Artery, inflammation, chronic active				1 (2%)
Seminal vesicle	(50)			(50)
Inflammation	1 (2%)			

TABLE C4
Summary of the Incidence of Nonneoplastic Lesions in Male Mice in the 2-Year Dermal Study
of Benzethonium Chloride (continued)

	Vehicle Control	0.15 mg/kg	0.5 mg/kg	1.5 mg/kg
2-Year Study (continued)				
Hematopoietic System				
Bone marrow	(50)			(50)
Erythroid cell, hyperplasia	4 (8%)			12 (24%)
Myeloid cell, hyperplasia	3 (6%)			4 (8%)
Lymph node, mandibular	(49)			(48)
Hyperplasia, lymphoid				1 (2%)
Lymph node, mesenteric	(48)			(47)
Hyperplasia, lymphoid				1 (2%)
Inflammation, granulomatous				1 (2%)
Spleen	(50)			(50)
Depletion lymphoid	3 (6%)			
Hematopoietic cell proliferation	11 (22%)			20 (40%)
Hyperplasia, lymphoid				1 (2%)
Inflammation, granulomatous				1 (2%)
Pigmentation, hemosiderin	1 (2%)			
Thymus	(31)			(39)
Depletion lymphoid	4 (13%)			3 (8%)
Hyperplasia, lymphoid				2 (5%)
Integumentary System				
Skin, control	(50)	(50)	(50)	(50)
Skin, site of application-no mass	(50)	(50)	(50)	(50)
Epithelial hyperplasia	2 (4%)	7 (14%)	16 (32%)	23 (46%)
Inflammation, chronic				2 (4%)
Ulcer	1 (2%)	1 (2%)	4 (8%)	2 (4%)
Sebaceous gland, hyperplasia			1 (2%)	
Musculoskeletal System				
None				
Nervous System				
Brain	(50)			(50)
Neuron, necrosis	2 (4%)			
Respiratory System				
Lung	(50)			(50)
Thrombosis				2 (4%)
Alveolar epithelium, hyperplasia	5 (10%)			3 (6%)
Bronchiole, hyperplasia				1 (2%)
Special Senses System				
Ear	(1)			(2)
Necrosis				1 (50%)
Eye	(1)			(1)
Cornea, inflammation	1 (100%)			1 (100%)
Harderian gland	(43)			(37)
Hyperplasia	2 (5%)			

TABLE C4
Summary of the Incidence of Nonneoplastic Lesions in Male Mice in the 2-Year Dermal Study
of Benzethonium Chloride (continued)

	Vehicle Control	0.15 mg/kg	0.5 mg/kg	1.5 mg/kg
2-Year Study (continued)				
Urinary System				
Kidney	(50)			(50)
Cyst				1 (2%)
Hydronephrosis	2 (4%)			1 (2%)
Nephropathy	48 (96%)			49 (98%)

^a Number of animals examined microscopically at the site and the number of animals with lesion

APPENDIX D
SUMMARY OF LESIONS IN FEMALE MICE
IN THE 2-YEAR DERMAL STUDY
OF BENZETHONIUM CHLORIDE

TABLE D1	Summary of the Incidence of Neoplasms in Female Mice	
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TABLE D1

Summary of the Incidence of Neoplasms in Female Mice in the 2-Year Dermal Study of Benzethonium Chloride^a

	Vehicle Control	0.15 mg/kg	0.5 mg/kg	1.5 mg/kg
Disposition Summary				
Animals initially in study	60	60	60	60
<i>15-Month interim evaluation</i>	8	7	10	6
Early deaths				
Accidental death	1			
Moribund	10	4	8	13
Natural deaths	3	15	9	7
Survivors				
Terminal sacrifice	38	34	31	34
Missexed			2	
Animals examined microscopically	60	60	58	60
15-Month Interim Evaluation				
Alimentary System				
Liver	(8)			(6)
Hepatocellular adenoma	1 (13%)			1 (17%)
Integumentary System				
Skin, control	(8)	(7)	(10)	(6)
Skin, site of application	(8)	(7)	(10)	(6)
Systems Examined With No Neoplasms Observed				
Cardiovascular System				
Endocrine System				
General Body System				
Genital System				
Hematopoietic System				
Musculoskeletal System				
Nervous System				
Respiratory System				
Special Senses System				
Urinary System				
2-Year Study				
Alimentary System				
Gallbladder	(52)			(53)
Histiocytic sarcoma	1 (2%)			
Intestine large, rectum	(52)			(53)
Liposarcoma, metastatic, skeletal muscle				1 (2%)
Intestine large, cecum	(52)			(53)
Intestine small, jejunum	(52)			(52)
Carcinoma	1 (2%)			
Hemangiosarcoma	1 (2%)			

TABLE D1

Summary of the Incidence of Neoplasms in Female Mice in the 2-Year Dermal Study of Benzethonium Chloride
(continued)

	Vehicle Control	0.15 mg/kg	0.5 mg/kg	1.5 mg/kg
2-Year Study (continued)				
Alimentary System (continued)				
Liver	(52)			(54)
Hemangioma				1 (2%)
Hemangiosarcoma	1 (2%)			
Hemangiosarcoma, metastatic, spleen	1 (2%)			
Hepatocellular carcinoma	7 (13%)			10 (19%)
Hepatocellular carcinoma, multiple	5 (10%)			1 (2%)
Hepatocellular adenoma	9 (17%)			12 (22%)
Hepatocellular adenoma, multiple	11 (21%)			6 (11%)
Hepatocholangiocarcinoma, multiple				1 (2%)
Histiocytic sarcoma	3 (6%)			
Plasma cell tumor malignant, metastatic, spleen	1 (2%)			
Mesentery	(10)			(9)
Hemangioma	1 (10%)			
Hepatocholangiocarcinoma, metastatic, liver				1 (11%)
Histiocytic sarcoma	1 (10%)			
Myxosarcoma, metastatic, skin	1 (10%)			
Pancreas	(52)			(53)
Histiocytic sarcoma	1 (2%)			
Leiomyosarcoma, metastatic, uterus				1 (2%)
Salivary glands	(52)			(54)
Stomach, forestomach	(51)			(53)
Cardiovascular System				
Heart	(52)			(54)
Plasma cell tumor malignant, metastatic, spleen	1 (2%)			
Endocrine System				
Adrenal cortex	(52)			(54)
Histiocytic sarcoma	1 (2%)			
Pituitary gland	(52)			(50)
Histiocytic sarcoma	1 (2%)			
Pars distalis, adenoma	6 (12%)			5 (10%)
Thyroid gland	(52)			(54)
Follicular cell, adenoma	3 (6%)			1 (2%)
Follicular cell, carcinoma	1 (2%)			
General Body System				
None				

TABLE D1

Summary of the Incidence of Neoplasms in Female Mice in the 2-Year Dermal Study of Benzethonium Chloride^a

	Vehicle Control	0.15 mg/kg	0.5 mg/kg	1.5 mg/kg
Disposition Summary				
Animals initially in study	60	60	60	60
<i>15-Month interim evaluation</i>	8	7	10	6
Early deaths				
Accidental death	1			
Moribund	10	4	8	13
Natural deaths	3	15	9	7
Survivors				
Terminal sacrifice	38	34	31	34
Missexed			2	
Animals examined microscopically	60	60	58	60
15-Month Interim Evaluation				
Alimentary System				
Liver	(8)			(6)
Hepatocellular adenoma	1 (13%)			1 (17%)
Integumentary System				
Skin, control	(8)	(7)	(10)	(6)
Skin, site of application	(8)	(7)	(10)	(6)
Systems Examined With No Neoplasms Observed				
Cardiovascular System				
Endocrine System				
General Body System				
Genital System				
Hematopoietic System				
Musculoskeletal System				
Nervous System				
Respiratory System				
Special Senses System				
Urinary System				
2-Year Study				
Alimentary System				
Gallbladder	(52)			(53)
Histiocytic sarcoma	1 (2%)			
Intestine large, rectum	(52)			(53)
Liposarcoma, metastatic, skeletal muscle				1 (2%)
Intestine large, cecum	(52)			(53)
Intestine small, jejunum	(52)			(52)
Carcinoma	1 (2%)			
Hemangiosarcoma	1 (2%)			

TABLE D1

Summary of the Incidence of Neoplasms in Female Mice in the 2-Year Dermal Study of Benzethonium Chloride
(continued)

	Vehicle Control	0.15 mg/kg	0.5 mg/kg	1.5 mg/kg
2-Year Study (continued)				
Genital System				
Ovary	(52)			(52)
Cystadenoma	3 (6%)			2 (4%)
Hemangioma				1 (2%)
Histiocytic sarcoma	2 (4%)			
Uterus	(52)			(53)
Carcinoma				1 (2%)
Histiocytic sarcoma	3 (6%)			1 (2%)
Leiomyosarcoma				1 (2%)
Polyp stromal	1 (2%)			1 (2%)
Hematopoietic System				
Blood				(1)
Bone marrow	(52)			(53)
Hemangiosarcoma, metastatic, spleen				3 (6%)
Histiocytic sarcoma	2 (4%)			
Plasma cell tumor malignant, metastatic, spleen	1 (2%)			
Lymph node	(7)			(2)
Lumbar, histiocytic sarcoma	1 (14%)			
Mediastinal, histiocytic sarcoma	1 (14%)			
Pancreatic, hepatocholangiocarcinoma, metastatic, liver				1 (50%)
Lymph node, mandibular	(52)			(54)
Hemangioma	1 (2%)			
Histiocytic sarcoma	3 (6%)			
Plasma cell tumor malignant, metastatic, spleen	1 (2%)			
Lymph node, mesenteric	(50)			(49)
Histiocytic sarcoma	3 (6%)			
Plasma cell tumor malignant, metastatic, spleen	1 (2%)			
Spleen	(52)			(53)
Hemangiosarcoma	1 (2%)			3 (6%)
Histiocytic sarcoma	1 (2%)			
Plasma cell tumor malignant	1 (2%)			
Thymus	(41)			(45)
Plasma cell tumor malignant, metastatic, spleen	1 (2%)			
Integumentary System				
Skin, control	(52)	(52)	(48)	(53)
Subcutaneous tissue, fibrosarcoma	2 (4%)			
Subcutaneous tissue, hemangiosarcoma			1 (2%)	
Subcutaneous tissue, sarcoma	1 (2%)			
Skin, site of application-no mass	(52)	(52)	(48)	(53)
Subcutaneous tissue, hemangioma	1 (2%)			
Skin, site of application-mass	(52)			(53)
Subcutaneous tissue, sarcoma	1 (2%)			1 (2%)

TABLE D1

Summary of the Incidence of Neoplasms in Female Mice in the 2-Year Dermal Study of Benzethonium Chloride
(continued)

	Vehicle Control	0.15 mg/kg	0.5 mg/kg	1.5 mg/kg
2-Year Study (continued)				
Musculoskeletal System				
Bone	(52)			(53)
Liposarcoma, metastatic, skeletal muscle				1 (2%)
Skeletal muscle				(4)
Hemangiosarcoma, metastatic, spleen				1 (25%)
Leiomyosarcoma, metastatic, uterus				1 (25%)
Liposarcoma				1 (25%)
Nervous System				
Spinal cord				(2)
Hemangiosarcoma, metastatic, spleen				1 (50%)
Respiratory System				
Lung	(52)			(54)
Alveolar/bronchiolar adenoma	1 (2%)			2 (4%)
Alveolar/bronchiolar carcinoma	1 (2%)			1 (2%)
Carcinoma, metastatic, harderian gland	1 (2%)			
Hepatocellular carcinoma, metastatic, liver	5 (10%)			3 (6%)
Hepatocholangiocarcinoma, metastatic, liver				1 (2%)
Histiocytic sarcoma	2 (4%)			
Liposarcoma, metastatic, skeletal muscle				1 (2%)
Plasma cell tumor malignant, metastatic, spleen	1 (2%)			
Special Senses System				
Harderian gland	(44)			(35)
Adenoma	2 (5%)			2 (6%)
Carcinoma	2 (5%)			1 (3%)
Zymbal's gland				(1)
Carcinoma				1 (100%)
Urinary System				
Kidney	(52)			(54)
Histiocytic sarcoma	3 (6%)			
Plasma cell tumor malignant, metastatic, spleen	1 (2%)			
Urinary bladder	(50)			(53)
Systemic Lesions				
Multiple organs ^b	(52)	(53)	(48)	(54)
Histiocytic sarcoma	3 (6%)			1 (2%)
Leukemia lymphocytic				1 (2%)
Lymphoma malignant lymphocytic	1 (2%)	1 (2%)		2 (4%)
Lymphoma malignant mixed	5 (10%)			3 (6%)
Lymphoma malignant undifferentiated cell	1 (2%)			

TABLE D1

Summary of the Incidence of Neoplasms in Female Mice in the 2-Year Dermal Study of Benzethonium Chloride
(continued)

	Vehicle Control	0.15 mg/kg	0.5 mg/kg	1.5 mg/kg
Neoplasm Summary				
Total animals with primary neoplasms ^c				
15-Month interim evaluation	1			1
2-Year study	41	1	1	42
Total primary neoplasms				
15-Month interim evaluation	1			1
2-Year study	74	1	1	62
Total animals with benign neoplasms				
15-Month interim evaluation	1			1
2-Year study	28			28
Total benign neoplasms				
15-Month interim evaluation	1			1
2-Year study	39			33
Total animals with malignant neoplasms				
2-Year study	27	1	1	27
Total malignant neoplasms				
2-Year study	35	1	1	29
Total animals with metastatic neoplasms				
2-Year study	9			8
Total metastatic neoplasms				
2-Year study	16			16

^a Number of animals examined microscopically at the site and the number of animals with neoplasm

^b Number of animals with any tissue examined microscopically

^c Primary neoplasms: all neoplasms except metastatic neoplasms

TABLE D2
Individual Animal Tumor Pathology of Female Mice in the 2-Year Dermal Study
of Benzethonium Chloride: Vehicle Control

	3	4	5	6	6	6	6	6	6	6	6	6	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7
Number of Days on Study	1	2	6	3	3	6	7	7	8	8	9	9	1	2	3	3	3	3	3	3	3	3	3	3	3	3	3
	7	8	8	2	9	0	4	7	7	9	1	8	9	2	2	2	2	2	2	2	2	2	2	2	2	2	3
Carcass ID Number	2	2	2	2	2	2	2	2	2	2	2	2	3	2	2	2	2	2	2	2	2	2	2	2	2	2	2
	6	8	9	8	5	6	8	8	5	5	4	9	0	6	4	4	4	4	4	4	5	5	5	5	5	5	5
	1	2	5	6	2	9	3	1	3	1	3	1	0	7	1	2	4	5	6	7	0	4	5	6	7		
Alimentary System																											
Esophagus	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Gallbladder	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Histiocytic sarcoma																											
Intestine large, colon	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Intestine large, rectum	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Intestine large, cecum	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Intestine small, duodenum	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Intestine small, jejunum	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Carcinoma																											
Hemangiosarcoma							X																				
Intestine small, ileum	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Liver	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Hemangiosarcoma							X																				
Hemangiosarcoma, metastatic, spleen											X																
Hepatocellular carcinoma									X			X							X						X		
Hepatocellular carcinoma, multiple															X									X			
Hepatocellular adenoma																											
Hepatocellular adenoma, multiple								X			X					X										X	X
Histiocytic sarcoma					X									X													
Plasma cell tumor malignant, metastatic, spleen																											
Mesentery	+	+		+														+		+							
Hemangioma																											

X: Lesion present
Blank: Not examined

TABLE D2
Individual Animal Tumor Pathology of Female Mice in the 2-Year Dermal Study
of Benzethonium Chloride: Vehicle Control (continued)

[illegible]

TABLE D2
Individual Animal Tumor Pathology of Female Mice in the 2-Year Dermal Study
of Benzethonium Chloride: Vehicle Control (continued)

[illegible]

[illegible]

[illegible]

TABLE D2[illegible]

Individual Animal Tumor Pathology of Female Mice in the 2-Year Dermal Study of Benzethonium Chloride: .15 mg/kg
(continued)

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Individual Animal Tumor Pathology of Female Mice in the 2-Year Dermal Study of Benzethonium Chloride: 0.5 mg/kg

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Individual Animal Tumor Pathology of Female Mice in the 2-Year Dermal Study of Benzethonium Chloride: 0.5 mg/kg
(continued)

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TABLE D2

Individual Animal Tumor Pathology of Female Mice in the 2-Year Dermal Study of Benzethonium Chloride: 1.5 mg/kg

Number of Days on Study	1	2	3	4	4	4	5	5	5	6	6	6	6	6	6	6	6	6	7	7	7	7	7	7	7	7
	2	4	1	2	7	8	2	5	8	1	4	8	8	9	9	9	9	9	9	2	2	2	2	2	2	2
	0	9	6	8	9	5	0	1	9	2	7	2	9	0	1	5	8	8	8	0	9	9	9	9	9	9
Carcass ID Number	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
	2	7	2	6	4	3	4	7	5	2	6	7	3	3	4	6	3	6	7	5	2	2	2	2	2	3
	7	7	9	0	9	1	1	6	0	3	1	2	8	3	5	9	5	2	9	8	1	2	5	6	8	0
Alimentary System																										
Esophagus	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Gallbladder	+	+	A	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Intestine large, colon	+	+	M	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Intestine large, rectum	+	+	M	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Liposarcoma, metastatic, skeletal muscle																										
Intestine large, cecum	+	+	M	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Intestine small, duodenum	+	+	M	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Intestine small, jejunum	+	M	M	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Intestine small, ileum	+	M	M	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Liver	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Hemangioma			X																							
Hepatocellular carcinoma						X		X				X							X	X						
Hepatocellular carcinoma, multiple													X													
Hepatocellular adenoma										X														X		X
Hepatocellular adenoma, multiple																									X	
Hepatocholangiocarcinoma, multiple																										
Mesentery																										
Hepatocholangiocarcinoma, metastatic, liver																										
Pancreas	+	+	M	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Leiomyosarcoma, metastatic, uterus																										
Salivary glands	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Stomach, forestomach	+	+	M	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Stomach, glandular	+	+	M	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Cardiovascular System																										
Heart	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Endocrine System																										
Adrenal cortex	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Adrenal medulla	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Islets, pancreatic	+	+	M	+	+	+	+	+	+	+	+	+	+	+	M	+	+	+	+	+	+	+	+	+	+	+
Parathyroid gland	+	+	M	+	+	+	+	+	+	+	+	+	+	+	M	M	+	+	+	+	+	+	+	+	M	+
Pituitary gland	+	+	M	+	+	+	+	M	+	+	+	+	+	+	M	+	+	M	+	+	+	+	+	+	+	+
Pars distalis, adenoma																										
Thyroid gland	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Follicular cell, adenoma																										
General Body System																										
None																										
Genital System																										
Clitoral gland																										
Ovary	+	+	M	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	M	+	+	+	+	+	+
Cystadenoma																										
Hemangioma																										

Individual Animal Tumor Pathology of Female Mice in the 2-Year Dermal Study of Benzethonium Chloride: 1.5 mg/kg
(continued)

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TABLE D2

Individual Animal Tumor Pathology of Female Mice in the 2-Year Dermal Study of Benzethonium Chloride: 1.5 mg/kg
(continued)

	1	2	3	4	4	4	5	5	5	6	6	6	6	6	6	6	6	6	6	7	7	7	7	7	7	7	7
Number of Days on Study	2	4	1	2	7	8	2	5	8	1	4	8	8	9	9	9	9	9	9	2	2	2	2	2	2	2	2
	0	9	6	8	9	5	0	1	9	2	7	2	9	0	1	5	8	8	8	0	9	9	9	9	9	9	9
Carcass ID Number	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
	2	7	2	6	4	3	4	7	5	2	6	7	3	3	4	6	3	6	7	5	2	2	2	2	2	3	3
	7	7	9	0	9	1	1	6	0	3	1	2	8	3	5	9	5	2	9	8	1	2	5	6	8	0	2
Genital System (continued)																											
Uterus	+	+	M	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Carcinoma																											
Histiocytic sarcoma																											
Leiomyosarcoma									X																		
Polyp stromal																X											
Hematopoietic System																											
Blood																			+								
Bone marrow	+	+	M	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Hemangiosarcoma, metastatic, spleen									X								X										
Lymph node																					+						
Pancreatic, hepatocholangiocarcinoma, metastatic, liver																							X				
Lymph node, mandibular	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Lymph node, mesenteric	+	+	M	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	M	+	+	M
Spleen	+	+	M	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Hemangiosarcoma									X								X										
Thymus	+	+	M	+	+	+	+	+	+	+	+	M	M	+	+	+	+	+	+	+	+	+	+	M	+	+	+
Integumentary System																											
Mammary gland	+	+	M	+	+	+	M	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Skin, control	+	+	A	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Skin, site of application-no mass	+	+	A	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Skin, site of application-mass																			+								
Subcutaneous tissue, sarcoma																	X										
Musculoskeletal System																											
Bone	+	+	M	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Liposarcoma, metastatic, skeletal muscle																	X										
Skeletal muscle									+	+	+						+										
Hemangiosarcoma, metastatic, spleen									X																		
Leiomyosarcoma, metastatic, uterus								X																			
Liposarcoma																	X										
Nervous System																											
Brain	+	+	M	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Peripheral nerve					+														+								
Spinal cord					+														+								
Hemangiosarcoma, metastatic, spleen																			X								

Individual Animal Tumor Pathology of Female Mice in the 2-Year Dermal Study of Benzethonium Chloride: 1.5 mg/kg
(continued)

TABLE D2

Individual Animal Tumor Pathology of Female Mice in the 2-Year Dermal Study of Benzethonium Chloride: 1.5 mg/kg
(continued)

	1	2	3	4	4	4	5	5	5	6	6	6	6	6	6	6	6	6	7	7	7	7	7	7	7	7
Number of Days on Study	2	4	1	2	7	8	2	5	8	1	4	8	8	9	9	9	9	9	9	2	2	2	2	2	2	2
	0	9	6	8	9	5	0	1	9	2	7	2	9	0	1	5	8	8	8	0	9	9	9	9	9	9
Carcass ID Number	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
	2	7	2	6	4	3	4	7	5	2	6	7	3	3	4	6	3	6	7	5	2	2	2	2	2	3
	7	7	9	0	9	1	1	6	0	3	1	2	8	3	5	9	5	2	9	8	1	2	5	6	8	0
Respiratory System																										
Lung	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Alveolar/bronchiolar adenoma						X																				
Alveolar/bronchiolar carcinoma																										
Hepatocellular carcinoma, metastatic, liver								X				X						X								
Hepatocholangiocarcinoma, metastatic, liver																					X					
Liposarcoma, metastatic, skeletal muscle															X											
Nose	+	+	M	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Trachea	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Special Senses System																										
Eye																										
Harderian gland	+	M	M	+	+	+	+	+	+	+	M	M	+	+	+	M	M	M	+	+	+	+	+	M	M	+
Adenoma																										X
Carcinoma																										
Zymbal's gland																										
Carcinoma																										
Urinary System																										
Kidney	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Urinary bladder	+	+	M	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Systemic Lesions																										
Multiple organs	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Histiocytic sarcoma																										
Leukemia lymphocytic																		X								
Lymphoma malignant lymphocytic											X															
Lymphoma malignant mixed		X								X																

Individual Animal Tumor Pathology of Female Mice in the 2-Year Dermal Study of Benzethonium Chloride: 1.5 mg/kg
(continued)

TABLE D3

Statistical Analysis of Primary Neoplasms in Female Mice in the 2-Year Dermal Study of Benzethonium Chloride

	Vehicle Control	0.15 mg/kg	0.5 mg/kg	1.5 mg/kg
Harderian Gland: Adenoma or Carcinoma				
Overall rate ^a	4/52 (8%)	— ^e	—	3/54 (6%)
Adjusted rate ^b	10.1%			8.8%
Terminal rate ^c	3/38 (8%)			3/34 (9%)
First incidence (days)	698			729 (T)
Life table test ^d				P=0.558N
Logistic regression test ^d				P=0.550N
Fisher exact test ^d				P=0.479N
Liver: Hepatocellular Adenoma				
Overall rate	20/52 (38%)	—	—	18/54 (33%)
Adjusted rate	49.7%			51.1%
Terminal rate	18/38 (47%)			17/34 (50%)
First incidence (days)	674			589
Life table test				P=0.584
Logistic regression test				P=0.573N
Fisher exact test				P=0.364N
Liver: Hepatocellular Carcinoma				
Overall rate	12/52 (23%)	—	—	11/54 (20%)
Adjusted rate	29.0%			26.2%
Terminal rate	9/38 (24%)			5/34 (15%)
First incidence (days)	677			485
Life table test				P=0.583
Logistic regression test				P=0.482N
Fisher exact test				P=0.459N
Liver: Hepatocellular Adenoma or Carcinoma				
Overall rate	27/52 (52%)	—	—	25/54 (46%)
Adjusted rate	62.6%			60.2%
Terminal rate	22/38 (58%)			18/34 (53%)
First incidence (days)	674			485
Life table test				P=0.531
Logistic regression test				P=0.512N
Fisher exact test				P=0.350N
Lung: Alveolar/Bronchiolar Adenoma or Carcinoma				
Overall rate	2/52 (4%)	—	—	3/54 (6%)
Adjusted rate	5.3%			7.8%
Terminal rate	2/38 (5%)			2/34 (6%)
First incidence (days)	729 (T)			479
Life table test				P=0.460
Logistic regression test				P=0.571
Fisher exact test				P=0.518

TABLE D3

Statistical Analysis of Primary Neoplasms in Female Mice in the 2-Year Dermal Study of Benzethonium Chloride
(continued)

	Vehicle Control	0.15 mg/kg	0.5 mg/kg	1.5 mg/kg
Ovary: Cystadenoma				
Overall rate	3/52 (6%)	—	—	2/52 (4%)
Adjusted rate	7.9%			5.9%
Terminal rate	3/38 (8%)			2/34 (6%)
First incidence (days)	729 (T)			729 (T)
Life table test				P=0.551N
Logistic regression test				P=0.551N
Fisher exact test				P=0.500N
Pituitary Gland (Pars Distalis): Adenoma				
Overall rate	6/52 (12%)	—	—	5/50 (10%)
Adjusted rate	15.8%			14.3%
Terminal rate	6/38 (16%)			4/34 (12%)
First incidence (days)	729 (T)			720
Life table test				P=0.578N
Logistic regression test				P=0.578N
Fisher exact test				P=0.528N
Skin, Control (Subcutaneous Tissue): Fibrosarcoma or Sarcoma				
Overall rate	3/52 (6%)	0/53 (0%)	0/48 (0%)	1/54 (2%)
Adjusted rate	7.6%	0.0%	0.0%	2.5%
Terminal rate	2/38 (5%)	0/34 (0%)	0/31 (0%)	0/34 (0%)
First incidence (days)	698	—	—	691
Life table test	P=0.461N	P=0.143N	P=0.156N	P=0.339N
Logistic regression test	P=0.452N	P=0.145N	P=0.141N	P=0.321N
Cochran-Armitage test	P=0.438N			
Fisher exact test		P=0.118N	P=0.137N	P=0.295N
Spleen: Hemangiosarcoma				
Overall rate	1/52 (2%)	—	—	3/53 (6%)
Adjusted rate	2.3%			7.5%
Terminal rate	0/38 (0%)			1/34 (3%)
First incidence (days)	689			589
Life table test				P=0.285
Logistic regression test				P=0.315
Fisher exact test				P=0.316
Thyroid Gland (Follicular Cell): Adenoma				
Overall rate	3/52 (6%)	—	—	1/54 (2%)
Adjusted rate	7.3%			2.9%
Terminal rate	2/38 (5%)			1/34 (3%)
First incidence (days)	660			729 (T)
Life table test				P=0.345N
Logistic regression test				P=0.327N
Fisher exact test				P=0.295N

TABLE D3

Statistical Analysis of Primary Neoplasms in Female Mice in the 2-Year Dermal Study of Benzethonium Chloride
(continued)

	Vehicle Control	0.15 mg/kg	0.5 mg/kg	1.5 mg/kg
Thyroid Gland (Follicular Cell): Adenoma or Carcinoma				
Overall rate	4/52 (8%)	-	-	1/54 (2%)
Adjusted rate	9.9%			2.9%
Terminal rate	3/38 (8%)			1/34 (3%)
First incidence (days)	660			729 (T)
Life table test				P=0.215N
Logistic regression test				P=0.202N
Fisher exact test				P=0.170N
All Organs: Hemangioma				
Overall rate	3/52 (6%)	-	-	2/54 (4%)
Adjusted rate	7.9%			4.4%
Terminal rate	3/38 (8%)			0/34 (0%)
First incidence (days)	729 (T)			316
Life table test				P=0.534N
Logistic regression test				P=0.419N
Fisher exact test				P=0.482N
All Organs: Hemangiosarcoma				
Overall rate	3/52 (6%)	-	-	3/54 (6%)
Adjusted rate	6.5%			7.5%
Terminal rate	0/38 (0%)			1/34 (3%)
First incidence (days)	660			589
Life table test				P=0.626
Logistic regression test				P=0.632N
Fisher exact test				P=0.643N
All Organs: Hemangioma or Hemangiosarcoma				
Overall rate	6/52 (12%)	-	-	5/54 (9%)
Adjusted rate	13.9%			11.6%
Terminal rate	3/38 (8%)			1/34 (3%)
First incidence (days)	660			316
Life table test				P=0.549N
Logistic regression test				P=0.416N
Fisher exact test				P=0.473N
All Organs: Malignant Lymphoma (Lymphocytic, Mixed, or Undifferentiated Cell Type)				
Overall rate	7/52 (13%)	-	-	5/54 (9%)
Adjusted rate	16.9%			11.8%
Terminal rate	5/38 (13%)			2/34 (6%)
First incidence (days)	660			249
Life table test				P=0.444N
Logistic regression test				P=0.307N
Fisher exact test				P=0.354N

TABLE D3

Statistical Analysis of Primary Neoplasms in Female Mice in the 2-Year Dermal Study of Benzethonium Chloride
(continued)

	Vehicle Control	0.15 mg/kg	0.5 mg/kg	1.5 mg/kg
All Organs: Histiocytic Sarcoma				
Overall rate	3/52 (6%)	—	—	1/54 (2%)
Adjusted rate	7.0%			2.9%
Terminal rate	1/38 (3%)			1/34 (3%)
First incidence (days)	568			729 (T)
Life table test				P=0.347N
Logistic regression test				P=0.291N
Fisher exact test				P=0.295N
All Organs: Malignant Lymphoma or Histiocytic Sarcoma				
Overall rate	10/52 (19%)	—	—	6/54 (11%)
Adjusted rate	23.0%			14.5%
Terminal rate	6/38 (16%)			3/34 (9%)
First incidence (days)	568			249
Life table test				P=0.280N
Logistic regression test				P=0.155N
Fisher exact test				P=0.185N
All Organs: Benign Neoplasms				
Overall rate	28/52 (54%)	—	—	29/54 (54%)
Adjusted rate	68.0%			71.9%
Terminal rate	25/38 (66%)			23/34 (68%)
First incidence (days)	660			316
Life table test				P=0.282
Logistic regression test				P=0.403
Fisher exact test				P=0.571N
All Organs: Malignant Neoplasms				
Overall rate	27/52 (52%)	—	—	27/54 (50%)
Adjusted rate	56.2%			55.9%
Terminal rate	17/38 (45%)			13/34 (38%)
First incidence (days)	568			249
Life table test				P=0.413
Logistic regression test				P=0.545N
Fisher exact test				P=0.499N
All Organs: Benign or Malignant Neoplasms				
Overall rate	41/52 (79%)	—	—	43/54 (80%)
Adjusted rate	85.4%			84.3%
Terminal rate	31/38 (82%)			26/34 (76%)
First incidence (days)	568			249
Life table test				P=0.214
Logistic regression test				P=0.406
Fisher exact test				P=0.555

TABLE D3

Statistical Analysis of Primary Neoplasms in Female Mice in the 2-Year Dermal Study of Benzethonium Chloride

(continued)

(T) Terminal sacrifice

- ^a Number of neoplasm-bearing animals/number of animals examined. Denominator is number of animals examined microscopically for adrenal gland, bone marrow, brain, clitoral gland, gallbladder, heart, kidney, larynx, liver, lung, nose, ovary, pancreas, parathyroid gland, pituitary gland, salivary gland, spleen, thyroid gland, and urinary bladder; for other tissues, denominator is number of animals necropsied.
- ^b Kaplan-Meier estimated neoplasm incidence at the end of the study after adjustment for intercurrent mortality
- ^c Observed incidence at terminal kill
- ^d Beneath the dosed group incidence are the P values corresponding to pairwise comparisons between the controls and that dosed group. The life table test regards neoplasms in animals dying prior to terminal kill as being (directly or indirectly) the cause of death. The logistic regression test regards these lesions as nonfatal. The Cochran-Armitage and Fisher exact tests compare directly the overall incidence rates. For all tests, a negative trend or a lower incidence in a dose group is indicated by N.
- ^e Organ was not examined at this dose level.
- ^f Not applicable; no neoplasm in animal group

TABLE D4

Summary of the Incidence of Nonneoplastic Lesions in Female Mice in the 2-Year Dermal Study of Benzethonium Chloride^a

	Vehicle Control	0.15 mg/kg	0.5 mg/kg	1.5 mg/kg
Disposition Summary				
Animals initially in study	60	60	60	60
<i>15-Month interim evaluation</i>	8	7	10	6
Early deaths				
Accidental death	1			
Moribund	10	4	8	13
Natural deaths	3	15	9	7
Survivors				
Terminal sacrifice	38	34	31	34
Missexed			2	
Animals examined microscopically	60	60	58	60
15-Month Interim Evaluation				
Alimentary System				
Liver	(8)			(6)
Basophilic focus				1 (17%)
Endocrine System				
Pituitary gland	(8)			(5)
Pars distalis, hyperplasia	2 (25%)			1 (20%)
Genital System				
Ovary	(8)			(6)
Cyst	2 (25%)			1 (17%)
Uterus	(8)			(6)
Hyperplasia, cystic	7 (88%)			4 (67%)
Hematopoietic System				
Lymph node				(1)
Pancreatic, hyperplasia				1 (100%)
Lymph node, mandibular	(8)			(6)
Hyperplasia				1 (17%)
Spleen	(8)			(6)
Hematopoietic cell proliferation				1 (17%)
Hyperplasia, lymphoid				1 (17%)
Thymus	(8)			(6)
Depletion lymphoid				1 (17%)
Integumentary System				
Skin, control	(8)	(7)	(10)	(6)
Skin, site of application-no mass	(8)	(7)	(10)	(6)
Epithelial hyperplasia			3 (30%)	4 (67%)
Respiratory System				
Lung	(8)			(6)
Alveolar epithelium, hyperplasia	1 (13%)			1 (17%)

TABLE D4

Summary of the Incidence of Nonneoplastic Lesions in Female Mice in the 2-Year Dermal Study of Benzethonium Chloride (continued)

	Vehicle Control	0.15 mg/kg	0.5 mg/kg	1.5 mg/kg
15-Month Interim Evaluation (continued)				
Urinary System				
Kidney	(8)			(6)
Nephropathy	2 (25%)			1 (17%)
Systems Examined With No Lesions Observed				
Cardiovascular System				
General Body System				
Musculoskeletal System				
Nervous System				
Special Senses System				
2-Year Study				
Alimentary System				
Gallbladder	(52)			(53)
Ulcer				1 (2%)
Intestine large, rectum	(52)			(53)
Artery, inflammation, chronic active				1 (2%)
Intestine small, duodenum	(51)			(53)
Erosion	2 (4%)			
Intestine small, ileum	(52)			(52)
Inflammation, acute				1 (2%)
Liver	(52)			(54)
Angiectasis	2 (4%)			1 (2%)
Basophilic focus	1 (2%)			2 (4%)
Clear cell focus	1 (2%)			1 (2%)
Developmental malformation	1 (2%)			
Eosinophilic focus	12 (23%)			17 (31%)
Fibrosis	1 (2%)			
Hematopoietic cell proliferation	2 (4%)			1 (2%)
Hemorrhage				1 (2%)
Inflammation, chronic active	3 (6%)			
Mixed cell focus	2 (4%)			3 (6%)
Necrosis	5 (10%)			
Bile duct, cyst				1 (2%)
Centrilobular, fatty change	1 (2%)			
Hepatocyte, hyperplasia	1 (2%)			1 (2%)
Mesentery	(10)			(9)
Inflammation, chronic active				1 (11%)
Inflammation, suppurative	2 (20%)			1 (11%)
Fat, necrosis	6 (60%)			5 (56%)
Pancreas	(52)			(53)
Atrophy	2 (4%)			5 (9%)
Atypia cellular	1 (2%)			2 (4%)
Inflammation, chronic active				1 (2%)
Duct, cyst				3 (6%)
Stomach, forestomach	(51)			(53)
Erosion	1 (2%)			
Hyperplasia	1 (2%)			2 (4%)

TABLE D4

Summary of the Incidence of Nonneoplastic Lesions in Female Mice in the 2-Year Dermal Study of Benzethonium Chloride (continued)

	Vehicle Control	0.15 mg/kg	0.5 mg/kg	1.5 mg/kg
2-Year Study (continued)				
Alimentary System (continued)				
Stomach, glandular	(51)			(53)
Erosion	1 (2%)			
Inflammation, acute				1 (2%)
Mineralization				1 (2%)
Cardiovascular System				
Heart	(52)			(54)
Degeneration				1 (2%)
Inflammation, chronic active	3 (6%)			
Mineralization	1 (2%)			
Artery, inflammation, chronic active	1 (2%)			
Endocrine System				
Adrenal cortex	(52)			(54)
Accessory adrenal cortical nodule	4 (8%)			3 (6%)
Hyperplasia	3 (6%)			1 (2%)
Capsule, hyperplasia, adenomatous	1 (2%)			
Adrenal medulla	(51)			(54)
Hyperplasia	4 (8%)			2 (4%)
Islets, pancreatic	(52)			(52)
Hyperplasia	2 (4%)			3 (6%)
Pituitary gland	(52)			(50)
Angiectasis	2 (4%)			
Pars distalis, hyperplasia	25 (48%)			29 (58%)
Thyroid gland	(52)			(54)
Follicle, cyst	3 (6%)			
Follicular cell, hyperplasia	20 (38%)			21 (39%)
General Body System				
None				
Genital System				
Clitoral gland				(1)
Duct, ectasia				1 (100%)
Ovary	(52)			(52)
Angiectasis				1 (2%)
Cyst	25 (48%)			16 (31%)
Cyst dermoid				1 (2%)
Inflammation, suppurative	2 (4%)			1 (2%)
Interstitial, hyperplasia	1 (2%)			2 (4%)
Uterus	(52)			(53)
Angiectasis	1 (2%)			1 (2%)
Cyst	1 (2%)			
Hyperplasia, cystic	32 (62%)			39 (74%)
Infiltration cellular, histiocyte	1 (2%)			
Inflammation, chronic active	1 (2%)			1 (2%)

TABLE D4

Summary of the Incidence of Nonneoplastic Lesions in Female Mice in the 2-Year Dermal Study of Benzethonium Chloride (continued)

	Vehicle Control	0.15 mg/kg	0.5 mg/kg	1.5 mg/kg
2-Year Study (continued)				
Hematopoietic System				
Bone marrow	(52)			(53)
Myelofibrosis	13 (25%)			13 (25%)
Erythroid cell, hyperplasia	9 (17%)			3 (6%)
Myeloid cell, hyperplasia	6 (12%)			4 (8%)
Lymph node	(7)			(2)
Mediastinal, necrosis	1 (14%)			
Lymph node, mandibular	(52)			(54)
Atrophy	1 (2%)			
Hematopoietic cell proliferation				1 (2%)
Hyperplasia, lymphoid	2 (4%)			1 (2%)
Lymph node, mesenteric	(50)			(49)
Atrophy	1 (2%)			
Hematopoietic cell proliferation	1 (2%)			2 (4%)
Hyperplasia, lymphoid	2 (4%)			
Spleen	(52)			(53)
Angiectasis	1 (2%)			
Depletion lymphoid	2 (4%)			
Hematopoietic cell proliferation	16 (31%)			23 (43%)
Hyperplasia, lymphoid	1 (2%)			
Hyperplasia, macrophage				1 (2%)
Thymus	(41)			(45)
Depletion lymphoid	5 (12%)			6 (13%)
Hyperplasia, lymphoid	3 (7%)			4 (9%)
Integumentary System				
Mammary gland	(52)			(52)
Hyperplasia	3 (6%)			2 (4%)
Skin, control	(52)	(52)	(48)	(53)
Epithelial hyperplasia			1 (2%)	
Inflammation, chronic		1 (2%)		
Skin, site of application-no mass	(52)	(52)	(48)	(53)
Epithelial hyperplasia	3 (6%)	7 (13%)	6 (13%)	22 (42%)
Inflammation, chronic	1 (2%)	2 (4%)		
Ulcer			2 (4%)	
Sebaceous gland, hyperplasia			1 (2%)	
Musculoskeletal System				
Bone	(52)			(53)
Osteopetrosis	1 (2%)			
Nervous System				
Brain	(52)			(53)
Hemorrhage	1 (2%)			
Hydrocephalus				1 (2%)
Neuron, necrosis	1 (2%)			1 (2%)
Peripheral nerve				(2)
Degeneration				2 (100%)

TABLE D4

Summary of the Incidence of Nonneoplastic Lesions in Female Mice in the 2-Year Dermal Study of Benzethonium Chloride (continued)

	Vehicle Control	0.15 mg/kg	0.5 mg/kg	1.5 mg/kg
2-Year Study (continued)				
Respiratory System				
Lung	(52)			(54)
Hemorrhage				1 (2%)
Alveolar epithelium, hyperplasia	2 (4%)			2 (4%)
Alveolus, pigmentation, hemosiderin	2 (4%)			2 (4%)
Pleura, infiltration cellular, lymphocyte	1 (2%)			
Pleura, inflammation	1 (2%)			
Special Senses System				
Eye	(4)			(2)
Degeneration	3 (75%)			
Cornea, inflammation	1 (25%)			1 (50%)
Harderian gland	(44)			(35)
Hyperplasia	1 (2%)			2 (6%)
Urinary System				
Kidney	(52)			(54)
Glomerulosclerosis	1 (2%)			1 (2%)
Nephropathy	31 (60%)			38 (70%)

^a Number of animals examined microscopically at the site and the number of animals with lesion

TABLE D4

Summary of the Incidence of Nonneoplastic Lesions in Female Mice in the 2-Year Dermal Study of Benzethonium Chloride (continued)

	Vehicle Control	0.15 mg/kg	0.5 mg/kg	1.5 mg/kg
2-Year Study (continued)				
Alimentary System (continued)				
Stomach, glandular	(51)			(53)
Erosion	1 (2%)			
Inflammation, acute				1 (2%)
Mineralization				1 (2%)
Cardiovascular System				
Heart	(52)			(54)
Degeneration				1 (2%)
Inflammation, chronic active	3 (6%)			
Mineralization	1 (2%)			
Artery, inflammation, chronic active	1 (2%)			
Endocrine System				
Adrenal cortex	(52)			(54)
Accessory adrenal cortical nodule	4 (8%)			3 (6%)
Hyperplasia	3 (6%)			1 (2%)
Capsule, hyperplasia, adenomatous	1 (2%)			
Adrenal medulla	(51)			(54)
Hyperplasia	4 (8%)			2 (4%)
Islets, pancreatic	(52)			(52)
Hyperplasia	2 (4%)			3 (6%)
Pituitary gland	(52)			(50)
Angiectasis	2 (4%)			
Pars distalis, hyperplasia	25 (48%)			29 (58%)
Thyroid gland	(52)			(54)
Follicle, cyst	3 (6%)			
Follicular cell, hyperplasia	20 (38%)			21 (39%)
General Body System				
None				
Genital System				
Clitoral gland				(1)
Duct, ectasia				1 (100%)
Ovary	(52)			(52)
Angiectasis				1 (2%)
Cyst	25 (48%)			16 (31%)
Cyst dermoid				1 (2%)
Inflammation, suppurative	2 (4%)			1 (2%)
Interstitial, hyperplasia	1 (2%)			2 (4%)
Uterus	(52)			(53)
Angiectasis	1 (2%)			1 (2%)
Cyst	1 (2%)			
Hyperplasia, cystic	32 (62%)			39 (74%)
Infiltration cellular, histiocyte	1 (2%)			
Inflammation, chronic active	1 (2%)			1 (2%)

APPENDIX E

GENETIC TOXICOLOGY

<i>SALMONELLA</i> MUTAGENICITY TEST PROTOCOL	E-2
CHINESE HAMSTER OVARY CELL CYTOGENETICS PROTOCOLS	E-2
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GENETIC TOXICOLOGY

***SALMONELLA* MUTAGENICITY TEST PROTOCOL**

Testing was performed as reported by Zeiger *et al.* (1987). Benzethonium chloride was sent to the laboratory as a coded aliquot from Radian Corporation (Austin, TX). It was incubated with the *Salmonella typhimurium* tester strains (TA98, TA100, TA1535, and TA1537) either in buffer or S9 mix (metabolic activation enzymes and cofactors from Aroclor 1254-induced male Sprague-Dawley rat or Syrian hamster liver) for 20 minutes at 37° C. Top agar supplemented with *l*-histidine and *d*-biotin was added, and the contents of the tubes were mixed and poured onto the surfaces of minimal glucose agar plates. Histidine-independent mutant colonies arising on these plates were counted following incubation for 2 days at 37° C.

Each trial consisted of triplicate plates of concurrent positive and negative controls and at least five doses of benzethonium chloride. The high dose was limited by toxicity. All positive trials were repeated under the conditions that elicited the positive response. If no positive responses were seen, all negative trials were repeated.

In this assay, a positive response is defined as a reproducible, dose-related increase in histidine-independent (revertant) colonies in any one strain/activation combination. An equivocal response is defined as an increase in revertants that is not dose related, not reproducible, or is of insufficient magnitude to support a determination of mutagenicity. A negative response is obtained when no increase in revertant colonies is observed following chemical treatment. There was no minimum percentage or fold increase required for a chemical to be judged positive or weakly positive.

CHINESE HAMSTER OVARY CELL CYTOGENETICS PROTOCOLS

Testing was performed as reported by Galloway *et al.* (1987). Benzethonium chloride was sent to the laboratory as a coded aliquot by Radian Corporation. It was tested in cultured Chinese hamster ovary (CHO) cells for induction of sister chromatid exchanges (SCEs) and chromosomal aberrations (Abs), both in the presence and absence of Aroclor 1254-induced male Sprague-Dawley rat liver S9 and cofactor mix. Cultures were handled under gold lights to prevent photolysis of bromodeoxyuridine-substituted DNA. Each test consisted of concurrent solvent and positive controls and of at least three doses of benzethonium chloride; the high dose was limited by toxicity. A single flask per dose was used.

Sister Chromatid Exchange Test: In the SCE test without S9, CHO cells were incubated for 26 hours with benzethonium chloride in McCoy's 5A medium supplemented with fetal bovine serum, *l*-glutamine, and antibiotics. Bromodeoxyuridine (BrdU) was added 2 hours after culture initiation. After 26 hours, the medium containing benzethonium chloride was removed and replaced with fresh medium plus BrdU and Colcemid, and incubation was continued for 2 hours. Cells were then harvested by mitotic shake-off, fixed, and stained with Hoechst 33258 and Giemsa. In the SCE test with S9, cells were incubated with benzethonium chloride, serum-free medium, and S9 for 2 hours. The medium was then removed and replaced with medium containing serum and BrdU and no benzethonium chloride and incubation proceeded for an additional 26 hours, with Colcemid present for the final 2 hours. Harvesting and staining were the same as for cells treated without S9. All slides were scored blind and those from a single test were read by the same person. Fifty second-division metaphase cells were scored for frequency of SCEs/cell from each dose level.

Statistical analyses were conducted on the slopes of the dose-response curves and the individual dose points (Galloway *et al.*, 1987). An SCE frequency 20% above the concurrent solvent control value was chosen as a statistically conservative positive response. The probability of this level of difference occurring by chance at one dose point is less than 0.01; the probability for such a chance occurrence at two dose points is less than 0.001. An increase of 20% or greater at any single dose was considered weak evidence of activity; increases at two or more doses resulted in a determination that the trial was positive. A statistically significant trend ($P < 0.005$) in the absence of any responses reaching 20% above background led to a call of equivocal.

Chromosomal Aberrations Test: In the Abs test without S9, cells were incubated in McCoy's 5A medium with benzethonium chloride for 12 hours; Colcemid was added and incubation continued for 2 hours. The cells were then harvested by mitotic shake-off, fixed, and stained with Giemsa. For the Abs test with S9, cells were treated with benzethonium chloride and S9 for 2 hours, after which the treatment medium was removed and the cells were incubated for 12 hours in fresh medium, with Colcemid present for the final 2 hours. Cells were harvested in the same manner as for the treatment without S9.

Cells were selected for scoring on the basis of good morphology and completeness of karyotype (21 ± 2 chromosomes). All slides were scored blind and those from a single test were read by the same person. One hundred first-division metaphase cells were scored at each dose level. Classes of aberrations included simple (breaks and terminal deletions), complex (rearrangements and translocations), and other (pulverized cells, despiralized chromosomes, and cells containing 10 or more aberrations).

Chromosomal aberration data are presented as percentage of cells with aberrations. To arrive at a statistical call for a trial, analyses were conducted on both the dose response curve and individual dose points. For a single trial, a statistically significant ($P \leq 0.05$) difference for one dose point and a significant trend ($P \leq 0.015$) were considered weak evidence for a positive response; significant differences for two or more doses indicated the trial was positive. A positive trend test in the absence of a statistically significant increase at any one dose resulted in an equivocal call (Galloway *et al.*, 1987). Ultimately, the trial calls were based on a consideration of the statistical analyses as well as the biological information available to the reviewers.

RESULTS

Benzethonium chloride (0.010 to 100 $\mu\text{g}/\text{plate}$) was not mutagenic in *Salmonella typhimurium* strains TA98, TA100, TA1535, or TA1537 when tested with a preincubation protocol, with or without Aroclor 1254-induced male Sprague-Dawley rat or Syrian hamster liver S9 (Zeiger *et al.*, 1987; Table E1). In cytogenetic tests with cultured Chinese hamster ovary cells, benzethonium chloride did not induce SCEs (Table E2) or Abs (Table E3), with or without S9. Although an increase in chromosomal aberrations was observed in each of the two trials conducted, these increases were not statistically significant or dose related. No cell cycle delay was noted in either the Abs test or the SCE test.

TABLE E1
Mutagenicity of Benzethonium Chloride in *Salmonella typhimurium*^a

Strain	Dose ($\mu\text{g}/\text{plate}$)	Revertants/plate ^b					
		-S9		+10% hamster S9		+10% rat S9	
		Trial 1	Trial 2	Trial 1	Trial 2	Trial 1	Trial 2
TA98	0.00	20 \pm 1.8	14 \pm 4.1	19 \pm 2.5	28 \pm 3.2	22 \pm 3.0	21 \pm 0.9
	0.01	21 \pm 0.7	7 \pm 1.2				
	0.03	18 \pm 1.8	6 \pm 0.7				
	0.10	19 \pm 2.1	7 \pm 0.3				
	0.30	18 \pm 0.9	10 \pm 1.8				
	1.00	22 \pm 1.5	10 \pm 4.2	15 \pm 1.9	28 \pm 4.1	21 \pm 2.0	17 \pm 1.9
	3.30			16 \pm 2.9	20 \pm 0.7	24 \pm 3.2	20 \pm 2.4
	10.00			16 \pm 1.7	20 \pm 1.3	24 \pm 1.5	16 \pm 1.8
	33.00			10 \pm 2.1	19 \pm 2.9	16 \pm 0.7	24 \pm 1.2
	100.00			7 \pm 1.2	21 \pm 0.9	18 \pm 0.7	18 \pm 3.5
Trial summary		Negative	Negative	Negative	Negative	Negative	Negative
Positive control		180 \pm 63.8	123 \pm 9.0	1,152 \pm 40.1	719 \pm 45.8	872 \pm 70.4	1,150 \pm 21.3
TA100	0.00	90 \pm 1.8	101 \pm 9.0	109 \pm 7.1	152 \pm 5.2	128 \pm 9.3	142 \pm 4.7
	0.01	87 \pm 0.3	92 \pm 3.8				
	0.03	85 \pm 4.2	107 \pm 0.3				
	0.10	82 \pm 5.2	103 \pm 5.3				
	0.30	94 \pm 4.7	99 \pm 4.3				
	1.00	92 \pm 5.0	66 \pm 1.7	105 \pm 1.5	130 \pm 5.9	109 \pm 7.4	122 \pm 8.0
	3.30			100 \pm 3.2	133 \pm 16.3	121 \pm 6.1	132 \pm 10.1
	10.00			94 \pm 2.3	140 \pm 14.2	118 \pm 13.0	144 \pm 6.9
	33.00			84 \pm 4.1	144 \pm 8.8	123 \pm 8.7	141 \pm 1.3
	100.00			34 \pm 3.8	143 \pm 15.2	128 \pm 6.0	toxic
Trial summary		Negative	Negative	Negative	Negative	Negative	Negative
Positive control ^c		936 \pm 11.1	1,024 \pm 18.1	1,636 \pm 125.5	1,021 \pm 63.2	1,264 \pm 205.4	2,105 \pm 62.1
TA1535	0.00	18 \pm 1.2	4 \pm 1.5	17 \pm 1.5	6 \pm 0.0	25 \pm 1.5	7 \pm 1.7
	0.01	14 \pm 1.9	4 \pm 0.7				
	0.03	16 \pm 3.7	4 \pm 1.2				
	0.10	14 \pm 3.2	5 \pm 0.7				
	0.30	8 \pm 1.5	2 \pm 1.2				
	1.00	8 \pm 0.6	2 \pm 0.6	18 \pm 3.3	4 \pm 1.8	16 \pm 0.9	5 \pm 1.9
	3.30			26 \pm 1.3	5 \pm 1.5	20 \pm 2.0	6 \pm 2.5
	10.00			15 \pm 4.2	3 \pm 0.9	17 \pm 1.5	5 \pm 1.7
	33.00			9 \pm 0.9	4 \pm 0.7	8 \pm 1.5	4 \pm 0.7
	100.00			toxic	toxic	toxic	toxic
Trial summary		Negative	Negative	Negative	Negative	Negative	Negative
Positive control		942 \pm 36.6	446 \pm 18.0	125 \pm 10.7	97 \pm 15.5	184 \pm 15.0	60 \pm 8.4

TABLE E1
Mutagenicity of Benzethonium Chloride in *Salmonella typhimurium* (continued)

Strain	Dose ($\mu\text{g}/\text{plate}$)	Revertants/plate					
		-S9		+10% hamster S9		+10% rat S9	
		Trial 1	Trial 2	Trial 1	Trial 2	Trial 1	Trial 2
TA1537	0.00	12 \pm 1.5	10 \pm 1.2	24 \pm 2.4	15 \pm 1.5	19 \pm 0.3	12 \pm 1.5
	0.01	9 \pm 0.6	13 \pm 0.7				
	0.03	10 \pm 2.8	9 \pm 1.5				
	0.10	14 \pm 1.0	7 \pm 0.9				
	0.30	16 \pm 0.7	7 \pm 2.3				
	1.00	11 \pm 3.8	7 \pm 1.2	22 \pm 2.4	13 \pm 1.0	23 \pm 3.0	12 \pm 1.5
	3.30			26 \pm 4.3	10 \pm 2.1	20 \pm 3.4	15 \pm 1.7
	10.00			14 \pm 0.6	9 \pm 2.4	13 \pm 2.0	7 \pm 2.1
	33.00			13 \pm 1.2	15 \pm 2.6	13 \pm 2.5	12 \pm 1.7
	100.00			1 \pm 0.7	13 \pm 2.5	toxic	toxic
	Trial summary	Negative	Negative	Negative	Negative	Negative	Negative
	Positive control	207 \pm 94.2	177 \pm 104.5	356 \pm 21.3	197 \pm 15.3	432 \pm 29.1	122 \pm 43.3

^a The study was performed at Case Western Reserve University. The detailed protocol and these data are presented in Zeiger *et al.* (1987). The high dose was limited by toxicity; 0 $\mu\text{g}/\text{plate}$ dose is the solvent control.

^b Revertants are presented as mean \pm standard error from three plates.

^c 2-Aminoanthracene was used on all strains in the presence of S9. In the absence of metabolic activation, 4-nitro-*o*-phenylenediamine was tested on TA98, sodium azide was tested on TA100 and TA1535, and 9-aminoacridine was tested on TA1537.

TABLE E2
Induction of Sister Chromatid Exchanges in Chinese Hamster Ovary Cells by Benzethonium Chloride^a

Compound	Dose ($\mu\text{g/mL}$)	Total Cells	No. of Chromo- somes	No. of SCEs	SCEs/ Chromo- some	SCEs/ Cell	Hrs in BrdU	Relative Change of SCEs/ Chromosome ^b (%)
-S9								
Summary: Negative								
Distilled water		50	1,047	434	0.41	8.7	26.0	
Mitomycin-C	0.005	25	523	639	1.22	25.6	26.0	194.76
Benzethonium chloride	0.960	50	1,049	458	0.43	9.2	26.0	5.33
	3.000	50	1,050	457	0.43	9.1	26.0	5.00
	9.600	50	1,048	473	0.45	9.5	26.0	8.88
								P=0.115 ^c
+S9								
Summary: Negative								
Distilled water		50	1,043	369	0.35	7.4	26.0	
Cyclophosphamide	1.00	50	1,045	790	0.75	15.8	26.0	113.69
Benzethonium chloride	3.00	50	1,048	347	0.33	6.9	26.0	-6.41
	9.60	50	1,050	359	0.34	7.2	26.0	-3.36
	30.00	50	1,047	367	0.35	7.3	26.0	-0.92
								P=0.495

^a Study performed at Columbia University. A detailed description of the protocol and these data are presented in Galloway *et al.* (1987). SCE=sister chromatid exchange; BrdU=bromodeoxyuridine.

^b SCEs/chromosome in treated cells versus SCEs/chromosome in solvent control cells $\times 100$.

^c Significance of relative SCEs/chromosome tested by the linear regression trend test vs. log of the dose

TABLE E3
Induction of Chromosomal Aberrations in Chinese Hamster Ovary Cells by Benzethonium Chloride^a

-S9					+S9				
Dose ($\mu\text{g/mL}$)	Total Cells	No. of Abs	Abs/ Cell	Percent Cells w/Abs	Dose ($\mu\text{g/mL}$)	Total Cells	No. of Abs	Abs/ Cell	Percent Cells w/Abs
Harvest time: 14.0 hours Summary: Negative					Harvest time: 14.0 hours Summary: Negative				
Distilled water	100	4	0.04	4.0	Distilled water	100	3	0.03	3.0
Mitomycin-C					Cyclophosphamide				
0.15	50	34	0.68	42.0	15.00	50	16	0.32	28.0
Benzethonium chloride					Benzethonium chloride				
0.96	100	11	0.11	10.0	3.00	100	5	0.05	5.0
3.00	100	10	0.10	10.0	9.60	100	6	0.06	5.0
9.60	100	8	0.08	8.0	30.00	100	6	0.06	6.0
P=0.162					P=0.172 ^b				

^a Study performed at Columbia University. The detailed protocol and these data are presented in Galloway *et al.* (1987).
 Abs=aberrations.

^b Significance of percent cells with aberrations tested by the linear regression trend test vs. log of the dose

APPENDIX F

ORGAN WEIGHTS

AND ORGAN-WEIGHT-TO-BODY-WEIGHT RATIOS

TABLE F1	Organ Weights and Organ-Weight-to-Body-Weight Ratios for Rats in the 16-Day Dermal Study of Benzethonium Chloride	F-2
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TABLE F1
Organ Weights and Organ-Weight-to-Body-Weight Ratios for Rats in the 16-Day Dermal Study
of Benzethonium Chloride^a

	Vehicle Control	6.3 mg/kg	12.5 mg/kg	25 mg/kg	50 mg/kg	100 mg/kg
n	5	5	5	5	5	5
Male						
Necropsy body wt	167 ± 7	165 ± 5	162 ± 7	154 ± 4	140 ± 6**	127 ± 6**
Brain						
Absolute	1.710 ± 0.008	1.685 ± 0.028	1.701 ± 0.017	1.671 ± 0.023	1.663 ± 0.012	1.647 ± 0.010*
Relative	10.30 ± 0.44	10.25 ± 0.18	10.57 ± 0.37	10.90 ± 0.32	11.93 ± 0.46*	13.11 ± 0.70**
Heart						
Absolute	0.686 ± 0.022	0.647 ± 0.016	0.637 ± 0.014	0.651 ± 0.016	0.631 ± 0.028	0.592 ± 0.014**
Relative	4.12 ± 0.14	3.93 ± 0.06	3.95 ± 0.11	4.24 ± 0.13	4.50 ± 0.11	4.72 ± 0.28*
R. Kidney						
Absolute	0.935 ± 0.034	0.916 ± 0.028	0.882 ± 0.030	0.817 ± 0.020**	0.806 ± 0.024**	0.780 ± 0.025**
Relative	5.60 ± 0.11	5.56 ± 0.06	5.46 ± 0.13	5.32 ± 0.10	5.77 ± 0.21	6.18 ± 0.25
Liver						
Absolute	10.880 ± 0.433	9.896 ± 0.421	9.485 ± 0.390*	9.093 ± 0.379**	8.794 ± 0.434**	7.881 ± 0.190**
Relative	65.14 ± 1.59	60.06 ± 1.18	58.67 ± 1.33	59.11 ± 1.38	62.65 ± 1.12	62.62 ± 3.04
Lungs						
Absolute	1.516 ± 0.131	1.301 ± 0.111	1.181 ± 0.026	1.518 ± 0.145	1.108 ± 0.064**	1.065 ± 0.028**
Relative	9.26 ± 1.20	7.85 ± 0.45	7.33 ± 0.24	9.98 ± 1.14	7.90 ± 0.35	8.45 ± 0.34
R. Testis						
Absolute	1.022 ± 0.023	0.956 ± 0.022	0.949 ± 0.018	0.907 ± 0.020	0.868 ± 0.075*	0.887 ± 0.066*
Relative	6.13 ± 0.16	5.82 ± 0.10	5.89 ± 0.20	5.92 ± 0.20	6.15 ± 0.37	6.97 ± 0.32*
Thymus						
Absolute	0.457 ± 0.018	0.410 ± 0.006	0.391 ± 0.032*	0.378 ± 0.024*	0.373 ± 0.015**	0.267 ± 0.017**
Relative	2.74 ± 0.08	2.50 ± 0.08	2.44 ± 0.24	2.48 ± 0.19	2.67 ± 0.13	2.12 ± 0.16*
Female						
Necropsy body wt	123 ± 3	120 ± 3	124 ± 2	116 ± 3	109 ± 5*	108 ± 6*
Brain						
Absolute	1.617 ± 0.029	1.611 ± 0.008	1.582 ± 0.029	1.570 ± 0.025	1.564 ± 0.033	1.543 ± 0.025
Relative	13.22 ± 0.17	13.45 ± 0.30	12.80 ± 0.18	13.61 ± 0.23	14.37 ± 0.42*	14.42 ± 0.56*
Heart						
Absolute	0.578 ± 0.009	0.567 ± 0.015	0.549 ± 0.013	0.549 ± 0.012	0.516 ± 0.018**	0.521 ± 0.007**
Relative	4.73 ± 0.12	4.74 ± 0.18	4.44 ± 0.13	4.76 ± 0.10	4.73 ± 0.06	4.88 ± 0.21
R. Kidney						
Absolute	0.677 ± 0.014	0.705 ± 0.013	0.690 ± 0.014	0.694 ± 0.027	0.691 ± 0.022	0.672 ± 0.026
Relative	5.53 ± 0.09	5.88 ± 0.09	5.58 ± 0.12	6.01 ± 0.09**	6.34 ± 0.15**	6.25 ± 0.13**
Liver						
Absolute	6.251 ± 0.234	6.532 ± 0.170	6.388 ± 0.195	6.463 ± 0.264	6.112 ± 0.212	5.964 ± 0.245
Relative	51.06 ± 1.47	54.42 ± 0.89	51.64 ± 1.28	55.99 ± 1.87*	56.06 ± 1.49*	55.45 ± 1.06*
Lungs						
Absolute	1.023 ± 0.035	1.062 ± 0.038	0.941 ± 0.038	0.958 ± 0.054	0.863 ± 0.077*	0.863 ± 0.058*
Relative	8.35 ± 0.18	8.87 ± 0.40	7.62 ± 0.32	8.28 ± 0.32	7.88 ± 0.57	7.99 ± 0.32
Thymus						
Absolute	0.358 ± 0.012	0.366 ± 0.019	0.374 ± 0.018	0.308 ± 0.016	0.273 ± 0.017**	0.249 ± 0.017**
Relative	2.93 ± 0.07	3.05 ± 0.13	3.02 ± 0.14	2.66 ± 0.10	2.49 ± 0.04**	2.30 ± 0.07**

* Significantly different ($P \leq 0.05$) from the control group by Williams' or Dunnett's test

** $P \leq 0.01$

^a Organ weights and body weights are given in grams; organ-weight-to-body-weight ratios are given as mg organ weight/g body weight (mean ± standard error).

TABLE F2
Organ Weights and Organ-Weight-to-Body-Weight Ratios for Rats in the 13-Week Dermal Study
of Benzethonium Chloride^a

	Vehicle Control	1.563 mg/kg	3.125 mg/kg	6.25 mg/kg	12.5 mg/kg	25 mg/kg
	10	10	10	10	10	10
Male						
Necropsy body wt	327 ± 6	326 ± 6	333 ± 6	323 ± 6	319 ± 5	287 ± 7**
Brain						
Absolute	1.900 ± 0.019	1.902 ± 0.021	1.952 ± 0.022	1.928 ± 0.017	1.933 ± 0.024	1.947 ± 0.019
Relative	5.82 ± 0.07	5.84 ± 0.06	5.87 ± 0.10	5.98 ± 0.09	6.07 ± 0.05	6.81 ± 0.15**
Heart						
Absolute	1.009 ± 0.022	1.014 ± 0.023	1.054 ± 0.023	1.029 ± 0.025	1.045 ± 0.019	1.048 ± 0.029
Relative	3.09 ± 0.03	3.11 ± 0.05	3.17 ± 0.06	3.18 ± 0.07	3.28 ± 0.07	3.67 ± 0.15**
Kidney						
Absolute	1.339 ± 0.033	1.318 ± 0.033	1.355 ± 0.026	1.309 ± 0.038	1.341 ± 0.023	1.353 ± 0.035
Relative	4.10 ± 0.05	4.04 ± 0.07	4.07 ± 0.06	4.05 ± 0.07	4.21 ± 0.09	4.72 ± 0.12**
Liver						
Absolute	16.102 ± 0.503	15.648 ± 0.660	16.669 ± 0.362	15.628 ± 0.424	15.629 ± 0.250	14.451 ± 0.400*
Relative	49.22 ± 1.02	47.86 ± 1.53	50.07 ± 0.80	48.29 ± 0.64	49.10 ± 0.98	50.39 ± 1.21
Lungs						
Absolute	2.030 ± 0.075	1.952 ± 0.069	2.058 ± 0.096	1.988 ± 0.055	1.914 ± 0.080	1.876 ± 0.034
Relative	6.20 ± 0.17	5.98 ± 0.17	6.17 ± 0.22	6.16 ± 0.17	6.00 ± 0.24	6.55 ± 0.15
R. Testis						
Absolute	1.452 ± 0.038	1.408 ± 0.022 ^b	1.486 ± 0.029	1.455 ± 0.016	1.485 ± 0.015	1.440 ± 0.032
Relative	4.45 ± 0.12	4.35 ± 0.09 ^b	4.47 ± 0.08	4.51 ± 0.06	4.66 ± 0.07	5.04 ± 0.16**
Thymus						
Absolute	0.301 ± 0.010	0.298 ± 0.013	0.301 ± 0.013	0.287 ± 0.016	0.275 ± 0.013	0.230 ± 0.008** ^b
Relative	0.92 ± 0.03	0.91 ± 0.03	0.91 ± 0.04	0.89 ± 0.04	0.86 ± 0.04	0.79 ± 0.03 ^b
Female						
Necropsy body wt	185 ± 3	186 ± 3	182 ± 2	183 ± 3	183 ± 2	181 ± 3
Brain						
Absolute	1.754 ± 0.019	1.771 ± 0.012	1.750 ± 0.018	1.736 ± 0.023	1.765 ± 0.021	1.733 ± 0.019
Relative	9.51 ± 0.18	9.55 ± 0.16	9.62 ± 0.11	9.51 ± 0.15	9.68 ± 0.16	9.59 ± 0.14
Heart						
Absolute	0.680 ± 0.010	0.722 ± 0.025	0.676 ± 0.007	0.674 ± 0.013	0.702 ± 0.009 ^b	0.687 ± 0.016
Relative	3.68 ± 0.05	3.87 ± 0.08	3.72 ± 0.03	3.69 ± 0.07	3.84 ± 0.07 ^b	3.80 ± 0.07
R. Kidney						
Absolute	0.770 ± 0.016	0.780 ± 0.018	0.783 ± 0.012	0.781 ± 0.015	0.785 ± 0.016	0.818 ± 0.013*
Relative	4.17 ± 0.05	4.19 ± 0.06	4.31 ± 0.06	4.27 ± 0.05	4.30 ± 0.07	4.52 ± 0.06**
Liver						
Absolute	7.212 ± 0.150	8.001 ± 0.217*	7.767 ± 0.218	7.434 ± 0.179	7.859 ± 0.219	7.660 ± 0.254
Relative	39.03 ± 0.67	43.00 ± 0.80*	42.75 ± 1.33	40.73 ± 1.04	43.03 ± 1.10*	42.33 ± 1.23
Lungs						
Absolute	1.346 ± 0.035	1.490 ± 0.072	1.346 ± 0.041	1.386 ± 0.052	1.401 ± 0.109	1.306 ± 0.039
Relative	7.29 ± 0.21	8.02 ± 0.39	7.39 ± 0.18	7.59 ± 0.28	7.70 ± 0.65	7.21 ± 0.16
Thymus						
Absolute	0.242 ± 0.007	0.242 ± 0.006	0.222 ± 0.011	0.228 ± 0.007	0.240 ± 0.007	0.232 ± 0.011
Relative	1.31 ± 0.04	1.30 ± 0.04	1.22 ± 0.05	1.25 ± 0.05	1.31 ± 0.04	1.29 ± 0.07

* Significantly different ($P \leq 0.05$) from the control group by Williams' or Dunnett's test

** $P \leq 0.01$

^a Organ weights and body weights are given in grams; organ-weight-to-body-weight ratios are given as mg organ weight/g body weight (mean ± standard error).

^b n=9

TABLE F3
Organ Weights and Organ-Weight-to-Body-Weight Ratios for Rats at the 15-Month Interim Evaluation
in the 2-Year Dermal Study of Benzethonium Chloride^a

	Vehicle Control	0.15 mg/kg	0.5 mg/kg	1.5 mg/kg
Male				
n	8	8	5	4
Necropsy body wt	498 ± 10	496 ± 18	520 ± 30	486 ± 12
L. Kidney				
Absolute	1.833 ± 0.032	1.929 ± 0.063	2.042 ± 0.139	1.835 ± 0.069
Relative	3.69 ± 0.09	3.90 ± 0.06	3.93 ± 0.13	3.78 ± 0.15
R. Kidney				
Absolute	1.823 ± 0.031	1.946 ± 0.061	2.017 ± 0.091	1.811 ± 0.070
Relative	3.67 ± 0.09	3.93 ± 0.07	3.90 ± 0.14	3.73 ± 0.11
Liver				
Absolute	19.236 ± 0.723	20.325 ± 1.167	21.970 ± 1.425	19.519 ± 0.510
Relative	38.73 ± 1.57	40.84 ± 1.28	42.31 ± 1.87	40.21 ± 0.73
Female				
n	9	7	9	7
Necropsy body wt	289 ± 8	289 ± 6	295 ± 9	285 ± 9
L. Kidney				
Absolute	1.158 ± 0.039	1.129 ± 0.033	1.215 ± 0.026	1.168 ± 0.045
Relative	4.01 ± 0.13	3.91 ± 0.11	4.14 ± 0.10	4.10 ± 0.15
R. Kidney				
Absolute	1.137 ± 0.034	1.142 ± 0.039	1.229 ± 0.034	1.139 ± 0.045
Relative	3.95 ± 0.13	3.95 ± 0.13	4.18 ± 0.06	4.01 ± 0.16
Uter				
Absolute	10.579 ± 0.324	10.849 ± 0.304	11.651 ± 0.581	10.434 ± 0.300
Relative	36.74 ± 1.32	37.54 ± 0.83	39.49 ± 1.27	36.64 ± 0.72

^a Organ weights and body weights are given in grams; organ-weight-to-body-weight ratios are given as mg organ weight/g body weight (mean ± standard error).

TABLE F4
Organ Weights and Organ-Weight-to-Body-Weight Ratios for Mice in the 16-Day Dermal Study of Benzethonium Chloride^a

	Vehicle Control	6.3 mg/kg	12.5 mg/kg	25 mg/kg	50 mg/kg	100 mg/kg
Male						
n	5	5	5	5	5	4
Necropsy body wt	25.1 ± 0.4	25.3 ± 0.6	25.9 ± 0.4	26.5 ± 0.5	26.4 ± 0.7	26.5 ± 0.5
Brain						
Absolute	0.472 ± 0.012	0.470 ± 0.004	0.475 ± 0.007	0.446 ± 0.014	0.455 ± 0.012	0.454 ± 0.007
Relative	18.83 ± 0.68	18.63 ± 0.45	18.37 ± 0.48	16.85 ± 0.55*	17.29 ± 0.74*	17.17 ± 0.23
Heart						
Absolute	0.154 ± 0.009	0.155 ± 0.010	0.154 ± 0.009	0.167 ± 0.006	0.155 ± 0.005	0.188 ± 0.017*
Relative	6.14 ± 0.39	6.16 ± 0.46	5.97 ± 0.46	6.31 ± 0.17	5.89 ± 0.23	7.06 ± 0.53
R. Kidney						
Absolute	0.285 ± 0.008	0.283 ± 0.005	0.273 ± 0.014	0.273 ± 0.008	0.262 ± 0.015	0.303 ± 0.003
Relative	11.34 ± 0.28	11.20 ± 0.23	10.55 ± 0.44	10.32 ± 0.32	9.94 ± 0.63	11.45 ± 0.25
Liver						
Absolute	1.663 ± 0.017	1.615 ± 0.047	1.689 ± 0.017	1.737 ± 0.062	1.687 ± 0.074	1.857 ± 0.035*
Relative	66.28 ± 1.32	63.99 ± 1.98	65.30 ± 0.60	65.57 ± 1.17	63.75 ± 1.46	70.21 ± 2.02
Lungs						
Absolute	0.241 ± 0.014 ^b	0.207 ± 0.006	0.230 ± 0.016	0.206 ± 0.003	0.222 ± 0.009	0.226 ± 0.019
Relative	9.64 ± 0.49 ^b	8.21 ± 0.27	8.88 ± 0.63	7.81 ± 0.18*	8.39 ± 0.24	8.50 ± 0.60
R. Testis						
Absolute	0.112 ± 0.005	0.115 ± 0.003	0.109 ± 0.005	0.106 ± 0.004	0.108 ± 0.005	0.112 ± 0.005
Relative	4.44 ± 0.17	4.55 ± 0.21	4.20 ± 0.15	4.00 ± 0.11	4.08 ± 0.13	4.21 ± 0.12
Thymus						
Absolute	0.052 ± 0.004	0.052 ± 0.003	0.051 ± 0.003	0.052 ± 0.004	0.041 ± 0.004	0.046 ± 0.001
Relative	2.07 ± 0.16	2.05 ± 0.14	1.96 ± 0.13	1.97 ± 0.17	1.54 ± 0.16*	1.72 ± 0.07
Female						
n	5	5	5	5	5	5
Necropsy body wt	21.2 ± 0.5	21.6 ± 0.4	21.0 ± 0.4	21.3 ± 0.3	21.1 ± 0.3	20.9 ± 0.5
Brain						
Absolute	0.460 ± 0.012	0.472 ± 0.007	0.455 ± 0.009	0.457 ± 0.004	0.440 ± 0.006	0.450 ± 0.005
Relative	21.71 ± 0.45	21.95 ± 0.62	21.69 ± 0.66	21.46 ± 0.18	20.87 ± 0.29	21.60 ± 0.44
Heart						
Absolute	0.119 ± 0.007	0.126 ± 0.004	0.123 ± 0.004	0.124 ± 0.002	0.125 ± 0.005	0.136 ± 0.006*
Relative	5.60 ± 0.23	5.85 ± 0.19	5.87 ± 0.25	5.84 ± 0.13	5.91 ± 0.22	6.54 ± 0.38*
R. Kidney						
Absolute	0.200 ± 0.008	0.190 ± 0.004	0.193 ± 0.003	0.193 ± 0.006	0.191 ± 0.006	0.206 ± 0.008
Relative	9.44 ± 0.25	8.83 ± 0.23	9.18 ± 0.22	9.06 ± 0.25	9.06 ± 0.22	9.87 ± 0.22
Liver						
Absolute	1.325 ± 0.016	1.334 ± 0.044	1.393 ± 0.048	1.347 ± 0.016	1.392 ± 0.028	1.401 ± 0.067
Relative	62.67 ± 1.30	61.81 ± 0.98	66.15 ± 1.49	63.36 ± 1.56	66.00 ± 0.73	66.97 ± 1.74*
Lungs						
Absolute	0.184 ± 0.007	0.213 ± 0.012	0.189 ± 0.006	0.196 ± 0.013	0.184 ± 0.004	0.186 ± 0.008
Relative	8.70 ± 0.21	9.88 ± 0.56	8.97 ± 0.21	9.23 ± 0.61	8.75 ± 0.27	8.87 ± 0.17
Thymus						
Absolute	0.069 ± 0.005	0.072 ± 0.003	0.066 ± 0.003	0.061 ± 0.005	0.061 ± 0.004	0.054 ± 0.006*
Relative	3.23 ± 0.19	3.32 ± 0.09	3.11 ± 0.10	2.88 ± 0.23	2.90 ± 0.18	2.59 ± 0.24*

* Significantly different ($P \leq 0.05$) from the control group by Williams' or Dunnett's test

^a Organ weights and body weights are given in grams; organ-weight-to-body-weight ratios are given as mg organ weight/g body weight (mean ± standard error).

^b n=4

TABLE F5

Organ Weights and Organ-Weight-to-Body-Weight Ratios for Mice in the 13-Week Dermal Study of Benzethonium Chloride^a

	Vehicle Control	1.563 mg/kg	3.125 mg/kg	6.25 mg/kg	12.5 mg/kg	25 mg/kg
n	10	10	10	10	10	10
Male						
Necropsy body wt	33.5 ± 1.0	32.6 ± 0.7	32.5 ± 1.0	32.4 ± 0.5	32.2 ± 0.8	31.4 ± 0.7
Brain						
Absolute	0.466 ± 0.007	0.454 ± 0.007	0.453 ± 0.009	0.472 ± 0.005	0.486 ± 0.007	0.465 ± 0.005
Relative	14.01 ± 0.46	13.98 ± 0.32	14.05 ± 0.41	14.61 ± 0.21	15.18 ± 0.31*	14.86 ± 0.23*
Heart						
Absolute	0.176 ± 0.005	0.176 ± 0.006	0.173 ± 0.007 ^b	0.183 ± 0.004	0.183 ± 0.007	0.180 ± 0.006
Relative	5.26 ± 0.18	5.40 ± 0.15	5.26 ± 0.13 ^b	5.68 ± 0.19	5.68 ± 0.13	5.77 ± 0.20*
R. Kidney						
Absolute	0.329 ± 0.011	0.323 ± 0.008	0.338 ± 0.012	0.339 ± 0.013	0.349 ± 0.007	0.346 ± 0.008
Relative	9.82 ± 0.24	9.95 ± 0.30	10.42 ± 0.28	10.46 ± 0.33	10.89 ± 0.21**	11.03 ± 0.17**
Liver						
Absolute	1.753 ± 0.054	1.684 ± 0.032	1.754 ± 0.058	1.764 ± 0.036	1.821 ± 0.052	1.786 ± 0.032
Relative	52.38 ± 1.23	51.86 ± 1.11	54.04 ± 1.00	54.50 ± 0.82	56.62 ± 0.61**	57.07 ± 0.99**
Lungs						
Absolute	0.283 ± 0.011	0.289 ± 0.017	0.281 ± 0.013	0.295 ± 0.010	0.273 ± 0.018	0.260 ± 0.016
Relative	8.53 ± 0.44	8.85 ± 0.40	8.62 ± 0.26	9.15 ± 0.40	8.43 ± 0.39	8.23 ± 0.41
R. Testis						
Absolute	0.117 ± 0.003	0.118 ± 0.003	0.116 ± 0.003	0.118 ± 0.002	0.114 ± 0.003	0.117 ± 0.002
Relative	3.48 ± 0.08	3.64 ± 0.08	3.56 ± 0.07	3.65 ± 0.09	3.55 ± 0.06	3.75 ± 0.03*
Thymus						
Absolute	0.042 ± 0.003	0.036 ± 0.002	0.038 ± 0.002	0.037 ± 0.002	0.038 ± 0.002	0.042 ± 0.001
Relative	1.27 ± 0.09	1.12 ± 0.06	1.16 ± 0.06	1.15 ± 0.06	1.17 ± 0.04	1.33 ± 0.05
Female						
Necropsy body wt	27.5 ± 0.8	27.5 ± 0.6	27.9 ± 0.6	27.8 ± 0.7	27.3 ± 0.7	27.8 ± 1.0
Brain						
Absolute	0.482 ± 0.007	0.464 ± 0.008	0.465 ± 0.009	0.473 ± 0.005	0.471 ± 0.008	0.484 ± 0.007
Relative	17.61 ± 0.42	16.96 ± 0.46	16.67 ± 0.32	17.08 ± 0.35	17.32 ± 0.48	17.57 ± 0.52
Heart						
Absolute	0.149 ± 0.007	0.144 ± 0.004	0.159 ± 0.008	0.169 ± 0.008	0.150 ± 0.006	0.155 ± 0.006
Relative	5.46 ± 0.28	5.25 ± 0.17	5.72 ± 0.31	6.08 ± 0.25	5.52 ± 0.21	5.62 ± 0.26
R. Kidney						
Absolute	0.217 ± 0.008	0.230 ± 0.006	0.227 ± 0.008	0.229 ± 0.005	0.219 ± 0.006	0.233 ± 0.006
Relative	7.92 ± 0.29	8.39 ± 0.26	8.13 ± 0.25	8.25 ± 0.19	8.03 ± 0.13	8.42 ± 0.14
Liver						
Absolute	1.491 ± 0.064	1.460 ± 0.048	1.510 ± 0.050	1.527 ± 0.039	1.522 ± 0.051	1.635 ± 0.050
Relative	54.32 ± 2.10	53.25 ± 1.82	54.00 ± 1.08	55.05 ± 1.30	55.75 ± 1.44	59.16 ± 1.84
Lungs						
Absolute	0.256 ± 0.012	0.250 ± 0.010	0.273 ± 0.016	0.261 ± 0.013	0.255 ± 0.009	0.250 ± 0.009
Relative	9.36 ± 0.46	9.10 ± 0.36	9.82 ± 0.64	9.37 ± 0.42	9.36 ± 0.33	9.07 ± 0.39
Thymus						
Absolute	0.051 ± 0.002	0.049 ± 0.002	0.048 ± 0.003	0.049 ± 0.003	0.050 ± 0.002	0.050 ± 0.003
Relative	1.85 ± 0.05	1.77 ± 0.07	1.72 ± 0.08	1.77 ± 0.07	1.81 ± 0.06	1.81 ± 0.07

* Significantly different ($P \leq 0.05$) from the control group by Williams' or Dunnett's test

** $P \leq 0.01$

^a Organ weights and body weights are given in grams; organ-weight-to-body-weight ratios are given as mg organ weight/g body weight (mean ± standard error).

^b n=9

TABLE F4
Organ Weights and Organ-Weight-to-Body-Weight Ratios for Mice in the 16-Day Dermal Study
of Benzethonium Chloride^a

	Vehicle Control	6.3 mg/kg	12.5 mg/kg	25 mg/kg	50 mg/kg	100 mg/kg
Male						
n	5	5	5	5	5	4
Necropsy body wt	25.1 ± 0.4	25.3 ± 0.6	25.9 ± 0.4	26.5 ± 0.5	26.4 ± 0.7	26.5 ± 0.5
Brain						
Absolute	0.472 ± 0.012	0.470 ± 0.004	0.475 ± 0.007	0.446 ± 0.014	0.455 ± 0.012	0.454 ± 0.007
Relative	18.83 ± 0.68	18.63 ± 0.45	18.37 ± 0.48	16.85 ± 0.55*	17.29 ± 0.74*	17.17 ± 0.23
Heart						
Absolute	0.154 ± 0.009	0.155 ± 0.010	0.154 ± 0.009	0.167 ± 0.006	0.155 ± 0.005	0.188 ± 0.017*
Relative	6.14 ± 0.39	6.16 ± 0.46	5.97 ± 0.46	6.31 ± 0.17	5.89 ± 0.23	7.06 ± 0.53
R. Kidney						
Absolute	0.285 ± 0.008	0.283 ± 0.005	0.273 ± 0.014	0.273 ± 0.008	0.262 ± 0.015	0.303 ± 0.003
Relative	11.34 ± 0.28	11.20 ± 0.23	10.55 ± 0.44	10.32 ± 0.32	9.94 ± 0.63	11.45 ± 0.25
Liver						
Absolute	1.663 ± 0.017	1.615 ± 0.047	1.689 ± 0.017	1.737 ± 0.062	1.687 ± 0.074	1.857 ± 0.035*
Relative	66.28 ± 1.32	63.99 ± 1.98	65.30 ± 0.60	65.57 ± 1.17	63.75 ± 1.46	70.21 ± 2.02
Lungs						
Absolute	0.241 ± 0.014 ^b	0.207 ± 0.006	0.230 ± 0.016	0.206 ± 0.003	0.222 ± 0.009	0.226 ± 0.019
Relative	9.64 ± 0.49 ^b	8.21 ± 0.27	8.88 ± 0.63	7.81 ± 0.18*	8.39 ± 0.24	8.50 ± 0.60
R. Testis						
Absolute	0.112 ± 0.005	0.115 ± 0.003	0.109 ± 0.005	0.106 ± 0.004	0.108 ± 0.005	0.112 ± 0.005
Relative	4.44 ± 0.17	4.55 ± 0.21	4.20 ± 0.15	4.00 ± 0.11	4.08 ± 0.13	4.21 ± 0.12
Thymus						
Absolute	0.052 ± 0.004	0.052 ± 0.003	0.051 ± 0.003	0.052 ± 0.004	0.041 ± 0.004	0.046 ± 0.001
Relative	2.07 ± 0.16	2.05 ± 0.14	1.96 ± 0.13	1.97 ± 0.17	1.54 ± 0.16*	1.72 ± 0.07
Female						
n	5	5	5	5	5	5
Necropsy body wt	21.2 ± 0.5	21.6 ± 0.4	21.0 ± 0.4	21.3 ± 0.3	21.1 ± 0.3	20.9 ± 0.5
Brain						
Absolute	0.460 ± 0.012	0.472 ± 0.007	0.455 ± 0.009	0.457 ± 0.004	0.440 ± 0.006	0.450 ± 0.005
Relative	21.71 ± 0.45	21.95 ± 0.62	21.69 ± 0.66	21.46 ± 0.18	20.87 ± 0.29	21.60 ± 0.44
Heart						
Absolute	0.119 ± 0.007	0.126 ± 0.004	0.123 ± 0.004	0.124 ± 0.002	0.125 ± 0.005	0.136 ± 0.006*
Relative	5.60 ± 0.23	5.85 ± 0.19	5.87 ± 0.25	5.84 ± 0.13	5.91 ± 0.22	6.54 ± 0.38*
R. Kidney						
Absolute	0.200 ± 0.008	0.190 ± 0.004	0.193 ± 0.003	0.193 ± 0.006	0.191 ± 0.006	0.206 ± 0.008
Relative	9.44 ± 0.25	8.83 ± 0.23	9.18 ± 0.22	9.06 ± 0.25	9.06 ± 0.22	9.87 ± 0.22
Liver						
Absolute	1.325 ± 0.016	1.334 ± 0.044	1.393 ± 0.048	1.347 ± 0.016	1.392 ± 0.028	1.401 ± 0.067
Relative	62.67 ± 1.30	61.81 ± 0.98	66.15 ± 1.49	63.36 ± 1.56	66.00 ± 0.73	66.97 ± 1.74*
Lungs						
Absolute	0.184 ± 0.007	0.213 ± 0.012	0.189 ± 0.006	0.196 ± 0.013	0.184 ± 0.004	0.186 ± 0.008
Relative	8.70 ± 0.21	9.88 ± 0.56	8.97 ± 0.21	9.23 ± 0.61	8.75 ± 0.27	8.87 ± 0.17
Thymus						
Absolute	0.069 ± 0.005	0.072 ± 0.003	0.066 ± 0.003	0.061 ± 0.005	0.061 ± 0.004	0.054 ± 0.006*
Relative	3.23 ± 0.19	3.32 ± 0.09	3.11 ± 0.10	2.88 ± 0.23	2.90 ± 0.18	2.59 ± 0.24*

* Significantly different ($P \leq 0.05$) from the control group by Williams' or Dunnett's test

^a Organ weights and body weights are given in grams; organ-weight-to-body-weight ratios are given as mg organ weight/g body weight (mean ± standard error).

^b n=4

TABLE F5

Organ Weights and Organ-Weight-to-Body-Weight Ratios for Mice in the 13-Week Dermal Study of Benzethonium Chloride^a

	Vehicle Control	1.563 mg/kg	3.125 mg/kg	6.25 mg/kg	12.5 mg/kg	25 mg/kg
n	10	10	10	10	10	10
Male						
Necropsy body wt	33.5 ± 1.0	32.6 ± 0.7	32.5 ± 1.0	32.4 ± 0.5	32.2 ± 0.8	31.4 ± 0.7
Brain						
Absolute	0.466 ± 0.007	0.454 ± 0.007	0.453 ± 0.009	0.472 ± 0.005	0.486 ± 0.007	0.465 ± 0.005
Relative	14.01 ± 0.46	13.98 ± 0.32	14.05 ± 0.41	14.61 ± 0.21	15.18 ± 0.31*	14.86 ± 0.23*
Heart						
Absolute	0.176 ± 0.005	0.176 ± 0.006	0.173 ± 0.007 ^b	0.183 ± 0.004	0.183 ± 0.007	0.180 ± 0.006
Relative	5.26 ± 0.18	5.40 ± 0.15	5.26 ± 0.13 ^b	5.68 ± 0.19	5.68 ± 0.13	5.77 ± 0.20*
R. Kidney						
Absolute	0.329 ± 0.011	0.323 ± 0.008	0.338 ± 0.012	0.339 ± 0.013	0.349 ± 0.007	0.346 ± 0.008
Relative	9.82 ± 0.24	9.95 ± 0.30	10.42 ± 0.28	10.46 ± 0.33	10.89 ± 0.21**	11.03 ± 0.17**
Liver						
Absolute	1.753 ± 0.054	1.684 ± 0.032	1.754 ± 0.058	1.764 ± 0.036	1.821 ± 0.052	1.786 ± 0.032
Relative	52.38 ± 1.23	51.86 ± 1.11	54.04 ± 1.00	54.50 ± 0.82	56.62 ± 0.61**	57.07 ± 0.99**
Lungs						
Absolute	0.283 ± 0.011	0.289 ± 0.017	0.281 ± 0.013	0.295 ± 0.010	0.273 ± 0.018	0.260 ± 0.016
Relative	8.53 ± 0.44	8.85 ± 0.40	8.62 ± 0.26	9.15 ± 0.40	8.43 ± 0.39	8.23 ± 0.41
R. Testis						
Absolute	0.117 ± 0.003	0.118 ± 0.003	0.116 ± 0.003	0.118 ± 0.002	0.114 ± 0.003	0.117 ± 0.002
Relative	3.48 ± 0.08	3.64 ± 0.08	3.56 ± 0.07	3.65 ± 0.09	3.55 ± 0.06	3.75 ± 0.03*
Thymus						
Absolute	0.042 ± 0.003	0.036 ± 0.002	0.038 ± 0.002	0.037 ± 0.002	0.038 ± 0.002	0.042 ± 0.001
Relative	1.27 ± 0.09	1.12 ± 0.06	1.16 ± 0.06	1.15 ± 0.06	1.17 ± 0.04	1.33 ± 0.05
Female						
Necropsy body wt	27.5 ± 0.8	27.5 ± 0.6	27.9 ± 0.6	27.8 ± 0.7	27.3 ± 0.7	27.8 ± 1.0
Brain						
Absolute	0.482 ± 0.007	0.464 ± 0.008	0.465 ± 0.009	0.473 ± 0.005	0.471 ± 0.008	0.484 ± 0.007
Relative	17.61 ± 0.42	16.96 ± 0.46	16.67 ± 0.32	17.08 ± 0.35	17.32 ± 0.48	17.57 ± 0.52
Heart						
Absolute	0.149 ± 0.007	0.144 ± 0.004	0.159 ± 0.008	0.169 ± 0.008	0.150 ± 0.006	0.155 ± 0.006
Relative	5.46 ± 0.28	5.25 ± 0.17	5.72 ± 0.31	6.08 ± 0.25	5.52 ± 0.21	5.62 ± 0.26
R. Kidney						
Absolute	0.217 ± 0.008	0.230 ± 0.006	0.227 ± 0.008	0.229 ± 0.005	0.219 ± 0.006	0.233 ± 0.006
Relative	7.92 ± 0.29	8.39 ± 0.26	8.13 ± 0.25	8.25 ± 0.19	8.03 ± 0.13	8.42 ± 0.14
Liver						
Absolute	1.491 ± 0.064	1.460 ± 0.048	1.510 ± 0.050	1.527 ± 0.039	1.522 ± 0.051	1.635 ± 0.050
Relative	54.32 ± 2.10	53.25 ± 1.82	54.00 ± 1.08	55.05 ± 1.30	55.75 ± 1.44	59.16 ± 1.84
Lungs						
Absolute	0.256 ± 0.012	0.250 ± 0.010	0.273 ± 0.016	0.261 ± 0.013	0.255 ± 0.009	0.250 ± 0.009
Relative	9.36 ± 0.46	9.10 ± 0.36	9.82 ± 0.64	9.37 ± 0.42	9.36 ± 0.33	9.07 ± 0.39
Thymus						
Absolute	0.051 ± 0.002	0.049 ± 0.002	0.048 ± 0.003	0.049 ± 0.003	0.050 ± 0.002	0.050 ± 0.003
Relative	1.85 ± 0.05	1.77 ± 0.07	1.72 ± 0.08	1.77 ± 0.07	1.81 ± 0.06	1.81 ± 0.07

* Significantly different ($P \leq 0.05$) from the control group by Williams' or Dunnett's test

** $P \leq 0.01$

^a Organ weights and body weights are given in grams; organ-weight-to-body-weight ratios are given as mg organ weight/g body weight (mean ± standard error).

^b n=9

TABLE F6
Organ Weights and Organ-Weight-to-Body-Weight Ratios for Mice at the 15-Month Interim Evaluation in the 2-Year Dermal Study of Benzethonium Chloride^a

	Vehicle Control	0.15 mg/kg	0.5 mg/kg	1.5 mg/kg
Male				
n	10	9	9	10
Necropsy body wt	51.2 ± 1.2	52.0 ± 1.4	49.6 ± 1.7	50.2 ± 0.8
L. Kidney				
Absolute	0.466 ± 0.014	0.436 ± 0.013	0.433 ± 0.010	0.453 ± 0.010
Relative	9.12 ± 0.31	8.40 ± 0.21	8.78 ± 0.23	9.05 ± 0.26
R. Kidney				
Absolute	0.485 ± 0.015	0.462 ± 0.011	0.450 ± 0.008	0.489 ± 0.010
Relative	9.48 ± 0.29	8.92 ± 0.20	9.11 ± 0.22	9.78 ± 0.26
Liver				
Absolute	3.296 ± 0.362	2.591 ± 0.142	2.978 ± 0.338	3.082 ± 0.358
Relative	65.51 ± 8.61	49.95 ± 2.65	61.70 ± 9.23	61.60 ± 7.37
Female				
n	8	7	10	6
Necropsy body wt	50.5 ± 1.5	52.3 ± 0.9	51.5 ± 2.5	51.1 ± 3.0
L. Kidney				
Absolute	0.280 ± 0.007	0.303 ± 0.016	0.296 ± 0.008	0.307 ± 0.013
Relative	5.55 ± 0.06	5.77 ± 0.24	5.82 ± 0.19	6.04 ± 0.17
R. Kidney				
Absolute	0.297 ± 0.009	0.320 ± 0.012	0.308 ± 0.009	0.322 ± 0.015
Relative	5.89 ± 0.10	6.12 ± 0.18	6.06 ± 0.25	6.35 ± 0.23
Liver				
Absolute	1.959 ± 0.074	2.061 ± 0.068 ^b	2.224 ± 0.090 ^{*c}	2.184 ± 0.102
Relative	38.82 ± 1.01	39.82 ± 0.85 ^b	42.09 ± 0.75 ^{*c}	42.95 ± 0.89 ^{**}

* Significantly different ($P \leq 0.05$) from the control group by Williams' or Dunnett's test

** $P \leq 0.01$

^a Organ weights and body weights are given in grams; organ-weight-to-body-weight ratios are given as mg organ weight/g body weight (mean ± standard error).

^b n=6

^c n=9

APPENDIX G

CHEMICAL CHARACTERIZATION AND DOSE FORMULATION STUDIES

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CHEMICAL CHARACTERIZATION AND DOSE FORMULATION STUDIES

PROCUREMENT AND CHARACTERIZATION OF BENZETHONIUM CHLORIDE

United States Pharmacopeia (USP) grade benzethonium chloride was obtained from Rohm and Haas (Philadelphia, PA) in one lot (W0061), which was used throughout the 16-day, 13-week, and 2-year dermal studies. Identity, purity, and stability analyses were conducted by the analytical chemistry laboratory, Midwest Research Institute (Kansas City, MO). Reports on analyses performed in support of the benzethonium chloride studies are on file at the National Institute of Environmental Health Sciences (NIEHS).

The chemical, a white powdered solid, was identified as benzethonium chloride by infrared, ultraviolet/visible, and nuclear magnetic resonance spectroscopy. All spectra were consistent with the literature spectra (*Sadtler Standard Spectra, Aldrich Library*) of benzethonium chloride (Figures G1 and G2).

The purity was determined by elemental analyses, Karl Fischer water analysis, functional group titration, thin-layer chromatography (TLC), and high performance liquid chromatography (HPLC). For functional group titration, samples were dissolved in 20 mL glacial acetic acid and 10 mL 2% mercury (II) acetate. The sample solutions were then titrated with 0.1 N perchloric acid and monitored potentiometrically using a combination pH/mV electrode filled with 3 M aqueous potassium chloride. TLC was performed on Silica Gel 60 F-254 plates using two solvent systems: A) *n*-butanol:water:glacial acetic acid (66:17:17), and B) acetone:concentrated ammonium hydroxide (90:10). Nicotinamide was used as a reference standard. Plates were examined under shortwave (254 nm) ultraviolet light and a spray of iodoplatinate reagent. High performance liquid chromatography was performed using a Waters μ Bondapak C₁₈ column using ultraviolet detection (280 nm) and a solvent system of 0.1 M methanesulfonic acid, in water, adjusted to pH 2.0 with 10 N sodium hydroxide:0.1 M methanesulfonic acid, in methanol, adjusted to pH 2.0 with 10 N sodium hydroxide (20:80). The flow rate was 1.0 mL/minute.

Elemental analyses for carbon, hydrogen, nitrogen, and chlorine were in agreement with the theoretical values for benzethonium chloride. Karl Fischer water analysis indicated $0.6\% \pm 0.3(s)\%$ water. Functional group titration indicated a purity of $98.5\% \pm 0.5\%$. Thin-layer chromatography by system A indicated a major spot and a trace impurity near the origin; system B indicated a major spot and a minor impurity near the origin. High performance liquid chromatography detected a major peak and no impurities greater than or equal to 0.1% of the major peak area. The overall purity was determined to be greater than 98%.

The analytical chemistry laboratory analyzed the chemical to determine if it met USP purity requirements. The complete battery of USP analyses was performed as a supplement to the chemical characterization of benzethonium chloride. The USP tests included a test for chloride, reaction with nitric acid and mercuric chloride, as well as reaction with sodium nitrite. Further tests included determination of melting point range, weight loss on drying, residue on ignition, and containment of ammonium compounds. The assay was a titration with sodium tetraphenylboron and a bromophenol blue indicator. The melting point range was 161.2° to 161.4° C, and the test for weight loss on drying yielded a value of $0.4\% \pm 0.3\%$ water. These values conform to the USP requirements for both analyses. The sample met USP specifications for the residue on ignition ($0.007\% \pm 0.002\%$) and ammonium compounds (no perceptible ammonium odor) tests. The titrimetric assay indicated that the sample contained $99.7\% \pm 0.05\%$ benzethonium chloride, which met USP requirements for purity.

Stability studies were performed by the analytical chemistry laboratory using the high performance liquid chromatography system previously described. These studies indicated that benzethonium chloride was stable as a bulk chemical for at least 2 weeks when stored protected from light at temperatures up to 60° C. The bulk chemical was stored at room temperature protected from light. The stability of the chemical was monitored periodically using HPLC methods similar to those previously described. No degradation of the bulk chemical was observed.

PREPARATION AND ANALYSIS OF DOSE FORMULATIONS

Dose formulation solutions were prepared by mixing benzethonium chloride and 95% ethanol (USP grade) to give the required concentrations (Table G1). The dose formulations were prepared once for the 16-day studies and every 2 weeks for the 13-week and 2-year studies and stored protected from light in sealed glass vials at room temperature. Dose formulations were discarded 3 weeks after the date of preparation.

Dose formulation stability studies were performed by the analytical chemistry laboratory. Aliquots of the 0.03 mg/mL formulation of benzethonium chloride were evaporated under nitrogen and redissolved in 5 mL of internal standard solution (octanophenone, 0.1 mg/mL in acetonitrile). HPLC was performed using a Chromanetics Licrosorb RP-2 column, with a flow rate of 2.0 mL/minute, a mobile phase of water:acetonitrile:glacial acetic acid (30:69:1), with octanophenone added as an internal standard and ultraviolet detection at 280 nm. The stability of the benzethonium chloride dose formulation was confirmed for at least 3 weeks at room temperature when stored protected from light, and for 3 hours when exposed to light and air.

Periodic analyses of the dose formulations of benzethonium chloride were conducted by the study laboratory and the analytical chemistry laboratory using ethanol dilutions and subsequent determination of absorbance at 227 nm. The study laboratory analyzed the dose formulations once during the 16-day studies (Table G2), three times during the 13-week studies (Table G3), and approximately every 2 months during the 2-year studies (Table G4). All of the dose formulations from the 16-day and 13-week studies were found to be within 10% of the target concentrations. In the 2-year study, 98% (169/173) of the dose formulations analyzed were within 10% of the target concentrations. Results of the periodic referee analyses performed by the analytical chemistry laboratory were in agreement with the results obtained by the study laboratory (Table G5).

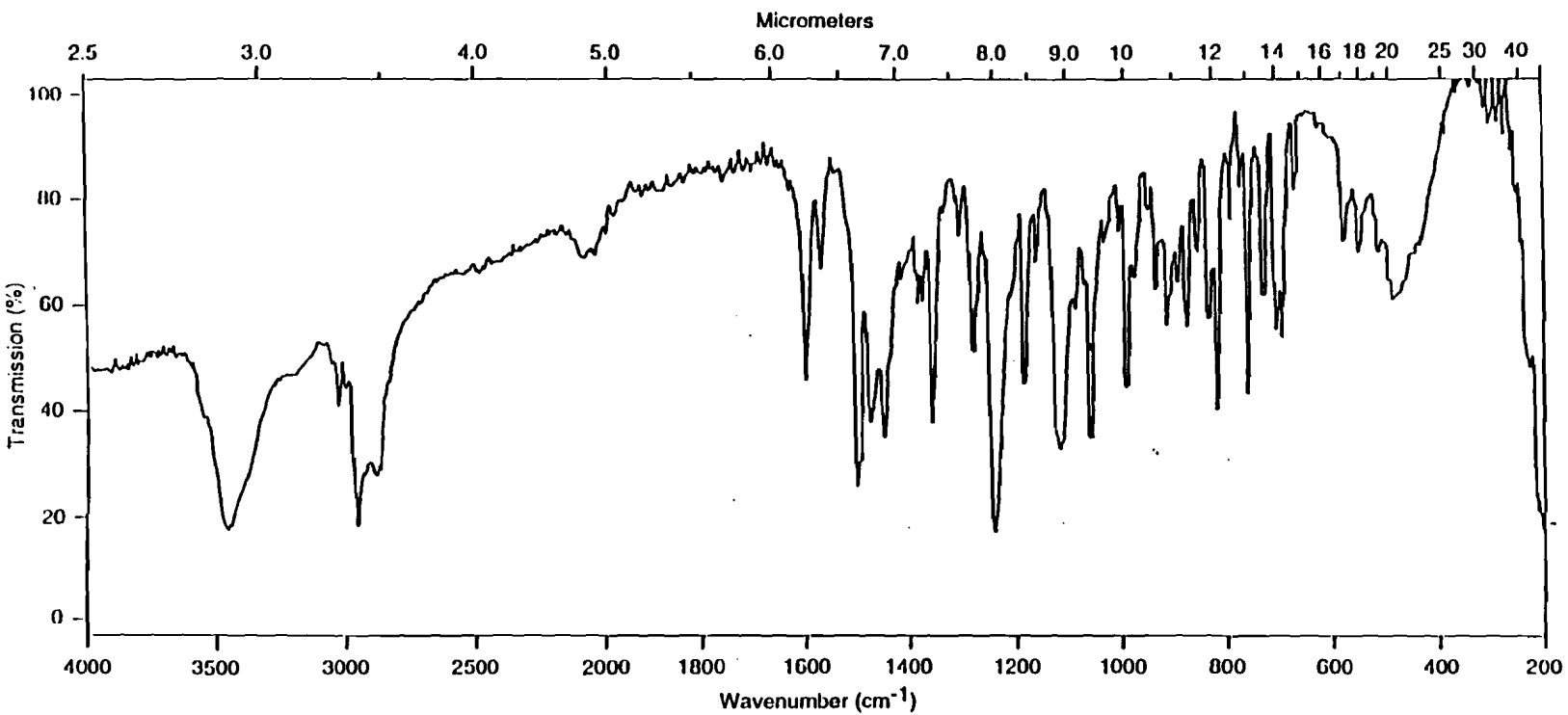


FIGURE G1
Infrared Absorption Spectrum of Benzethonium Chloride

SAMPLE	REMARKS	SOLVENT	ABSCISSA		ORDINATE		PERKIN ELMER®	
Benzethonium chloride	Transmittance curve used as reference beam	-	REP. SCAN	oil EXPANSION	1	EXPANSION	1	CHART NO. 203-1251
Lot No. JND001		CONCENTRATION 1% in KBr pellet	HIGH LIMIT	-	SUPPRESSION oil	%	10-100	OPERATOR A. Clark DATE 6/29/83
Batch No. 01		CELL PATH	LOW LIMIT	-	TIME DRIVE oil	SINGLE DEAM	-	ANS -
ORIGIN KBr pellet		REFERENCE				PRE SAMPLE CHOPPER	-	REF. NO. 235N DS 743

313MTR013P35 NMR 24M 09-09C M3

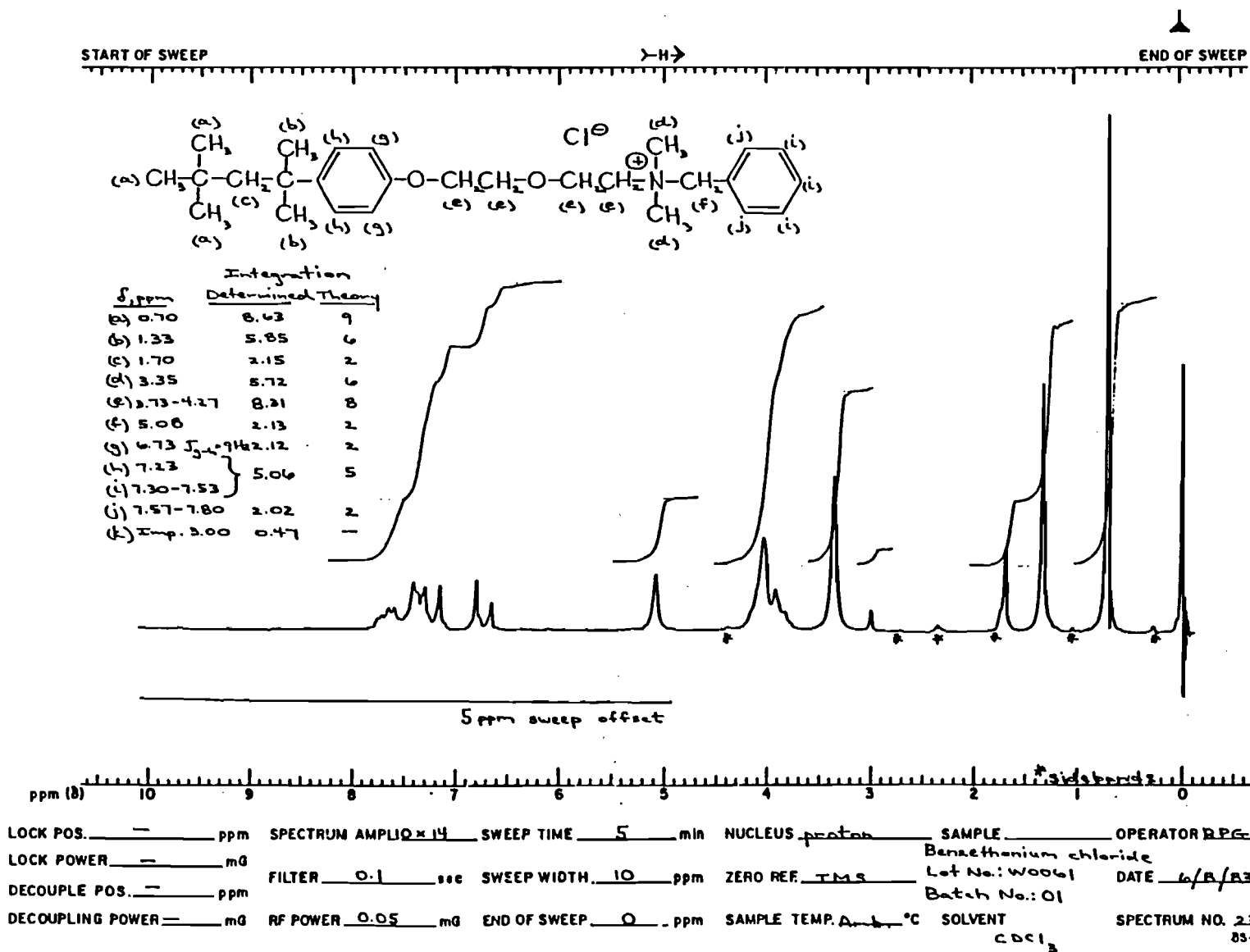


FIGURE G2
Nuclear Magnetic Resonance Spectrum of Benzethonium Chloride

TABLE G1

Preparation and Storage of Dose Formulations in the Dermal Studies of Benzethonium Chloride

16-Day Studies	13-Week Studies	2-Year Studies
Preparation		
Benzethonium chloride was weighed and transferred to a graduated cylinder and 95% ethanol was added to obtain the desired volume	Same as 16-day studies	Same as 16-day studies
Chemical Lot Number		
W0061	Same as 16-day studies	Same as 16-day studies
Maximum Storage Time		
3 weeks	Same as 16-day studies	Same as 16-day studies
Storage Conditions		
Stored at room temperature in sealed vials, protected from light	Same as 16-day studies	Same as 16-day studies
Study Laboratory		
Battelle Columbus Laboratories (Columbus, OH)	Same as 16-day studies	Same as 16-day studies
Referee Laboratory		
Midwest Research Institute (Kansas City, MO)	Same as 16-day studies	Same as 16-day studies

TABLE G2
Results of Analysis of Dose Formulations Administered to Rats and Mice
in the 16-Day Dermal Studies of Benzethonium Chloride

Date Prepared	Date Analyzed	Target Concentration (mg/mL) ^a	Determined Concentration ^b (mg/mL)	% Difference from Target
Rats				
Males				
12 December 1984	13 December 1984	6.0	5.93	-1
		12.0	11.9	-1
		24.0	23.6	-2
		48.0	47.0	-2
		96.0	94.8	-1
	27 December 1984 ^c	6.0	6.44	+7
		12.0	12.7	+6
		24.0	25.0	+4
		48.0	49.6	+3
Females				
12 December 1984	13 December 1984	4.0	4.07	+2
		8.0	8.07	+1
		16.0	15.9	-1
		32.0	30.6	-4
		64.0	63.1	-1
	27 December 1984 ^c	4.0	4.37	+9
		8.0	8.77	+10
		16.0	16.9	+6
		32.0	33.7	+5
		64.0	66.4	+4
	96.0	97.7	+2	

TABLE G2

Results of Analysis of Dose Formulations Administered to Rats and Mice
in the 16-Day Dermal Studies of Benzethonium Chloride (continued)

Date Prepared	Date Analyzed	Target Concentration (mg/mL) ^a	Determined Concentration ^b (mg/mL)	% Difference from Target
Mice				
12 December 1984	13 December 1984	1.5	1.55	+3
		3.0	3.05	+2
		6.0	6.06	+1
		12.0	12.1	+1
		24.0	23.6	-2
	27 December 1984 ^c	1.5	1.57	+5
		3.0	3.20	+7
		6.0	6.57	+10
		12.0	12.7	+6
		24.0	25.0	+4

^a Dosing volume = 0.250 mL (rats) or 0.100 mL (mice). For male rats, 6.0 mg/mL=6.3 mg/kg; 12.0 mg/mL=12.5 mg/kg; 24.0 mg/mL=25 mg/kg; 48 mg/mL=50 mg/kg; 96 mg/mL=100 mg/kg. For female rats, 4.0 mg/mL=6.3 mg/kg; 8.0 mg/mL=12.5 mg/kg; 16.0 mg/mL=25 mg/kg; 32 mg/mL=50 mg/kg; 64 mg/mL=100 mg/kg. For mice, 1.5 mg/mL=6.3 mg/kg; 3.0 mg/mL=12.5 mg/kg; 6.0 mg/mL=25 mg/kg; 12 mg/mL=50 mg/kg; 24 mg/mL=100 mg/kg.

^b Results of duplicate analyses

^c Animal room samples

TABLE G3
Results of Analysis of Dose Formulations Administered to Rats and Mice
in the 13-Week Dermal Studies of Benzethonium Chloride^a

Date Prepared	Date Analyzed	Target Concentration (mg/mL)	Determined Concentration (mg/mL) ^b	% Difference from Target
Rats				
25 April 1985	29 April 1985	1.563	1.58	+1
		3.125	3.04	-4
		6.25	6.41	+3
		12.5	12.7	+2
		25.0	26.0	+4
	14 May 1985 ^c	1.563	1.60	+2
		3.125	3.13	-1
		6.25	6.39	+2
		12.5	12.9	+3
		25.0	25.6	+2
	6 June 1985	1.563	1.59	+2
		3.125	3.10	-1
		6.25	6.38	+2
		12.5	13.0	+4
		25.0	25.9	+4
	26 June 1985 ^c	1.563	1.60	+2
		3.125	3.24	+4
		6.25	6.47	+4
		12.5	13.1	+5
		25.0	26.6	+6

TABLE G3
Results of Analysis of Dose Formulations Administered to Rats and Mice
in the 13-Week Dermal Studies of Benzethonium Chloride (continued)

Date Prepared	Date Analyzed	Target Concentration (mg/mL)	Determined Concentration (mg/mL)	% Difference from Target	
Rats (continued)					
18 July 1985	19 July 1985	1.563	1.55	-1	
		3.125	3.03	-3	
		6.25	6.12	-2	
		12.5	12.2	-2	
		25.0	24.5	-2	
		50.0	50.4	+1	
	5 August 1985 ^c	1.563	1.60	+2	
		3.175	3.24	+2	
		6.25	6.27	0	
		12.5	12.6	+1	
		25.0	25.7	+3	
	Mice				
	25 April 1985	29 April 1985	0.5	0.52	+4
1.0			0.96	-4	
2.0			2.02	+1	
4.0			4.00	0	
8.0			8.05	+1	
14 May 1985 ^c		0.5	0.519	+4	
		1.0	1.00	0	
		2.0	1.99	-1	
		4.0	4.03	+1	
		8.0	8.05	+1	
6 June 1985		7 June 1985	4.0	4.33	+8
		10 June 1985	8.0	8.35	+4

TABLE G3

Results of Analysis of Dose Formulations Administered to Rats and Mice
in the 13-Week Dermal Studies of Benzethonium Chloride (continued)

Date Prepared	Date Analyzed	Target Concentration (mg/mL)	Determined Concentration (mg/mL)	% Difference from Target
Mice (continued)				
6 June 1985	26 June 1985 ^d	4.0	4.38	+10
		8.0	8.28	+4
10 June 1985 ^e	10 June 1985	0.5	0.486	-3
		1.0	1.03	+3
		2.0	1.94	-3
10 June 1985	26 June 1985	0.5	0.50	0
		1.0	1.04	+4
		2.0	2.06	+3
18 July 1985	19 July 1985	0.5	0.48	-4
		1.0	0.97	-3
		2.0	2.00	0
		4.0	3.93	-2
		8.0	7.93	-1
		16.0	15.9	-1
	5 August 1985	0.5	0.487	-3
		1.0	1.01	+1
		2.0	2.00	0
		4.0	4.22	+6
		8.0	8.16	+2
		16.0	16.1	+1

^a The dosing volume was adjusted weekly following mean body weight measurements, but did not exceed 300 μ L for rats or 100 μ L for mice. For rats, 1.563 mg/mL=1.563 mg/kg; 3.125 mg/mL=3.125 mg/kg; 6.25 mg/mL=6.25 mg/kg; 12.5 mg/mL=12.5 mg/kg; 25 mg/mL=25 mg/kg. For mice, 0.5 mg/mL=1.563 mg/kg; 1 mg/mL=3.125 mg/kg; 2 mg/mL=6.25 mg/kg; 4 mg/mL=12.5 mg/kg; 8 mg/mL=25 mg/kg.

^b Results of duplicate analyses

^c Animal room samples

^d Result is average of analysis of three samples

^e Because of an interfering static charge on the prep balance, samples originally mixed on 6 June 1985 were remixed and reanalyzed on 10 June 1985.

TABLE G4
Results of Analysis of Dose Formulations Administered to Rats and Mice
in the 2-Year Dermal Studies of Benzethonium Chloride

Date Prepared	Date Analyzed	Target Concentration ^a (mg/mL)	Determined Concentration ^b (mg/mL)	% Difference from Target
Rats				
9 June 1987	11 June 1987	0.15	0.149 ^c	-1
		0.25	0.249 ^c	0
		0.5	0.497	-1
		0.83	0.829	0
		1.5	1.52	+1
		2.5	2.51	0
	23 June 1987 ^d	0.15	0.170 ^c	+13
		0.25	0.256 ^c	+2
		0.5	0.510	+2
		0.83	0.842	+1
		1.5	1.53	+2
		2.5	2.58	+3
	24 June 1987 ^d	0.15	0.154 ^c	+3
5 August 1987	6 August 1987	0.15	0.149 ^c	-1
		0.25	0.248 ^c	-1
		0.5	0.507	+1
		0.83	0.834	0
		1.5	1.55	+3
		2.5	2.54	+2
30 September 1987	1 October 1987	0.5	0.489	-2
		0.83	0.819	-1
		1.5	1.52	+1
		2.5	2.50	0
	7 October 1987	0.15	0.141	-6
		0.25	0.238	-5
	10 December 1987	0.15	0.145	-3
		0.25	0.246	-2
		0.5	0.497	-1
		0.83	0.817	-2
		1.5	1.49	-1
		2.5	2.49	0
9 December 1987	4 January 1988 ^d	0.15	0.166	+11
		0.25	0.277	+11
		0.5	0.537	+7
		0.83	0.870	+5
		1.5	1.59	+6
		2.5	2.66	+6
	21 January 1988	0.15	0.146	-3
		0.25	0.246	-2
		0.5	0.502	0
		0.83	0.827	0
20 January 1988	21 January 1988	1.5	1.52	+1
		2.5	2.51	0

TABLE G3

Results of Analysis of Dose Formulations Administered to Rats and Mice
in the 13-Week Dermal Studies of Benzethonium Chloride (continued)

Date Prepared	Date Analyzed	Target Concentration (mg/mL)	Determined Concentration (mg/mL)	% Difference from Target
Mice (continued)				
6 June 1985	26 June 1985 ^d	4.0	4.38	+10
		8.0	8.28	+4
10 June 1985 ^e	10 June 1985	0.5	0.486	-3
		1.0	1.03	+3
		2.0	1.94	-3
10 June 1985	26 June 1985	0.5	0.50	0
		1.0	1.04	+4
		2.0	2.06	+3
18 July 1985	19 July 1985	0.5	0.48	-4
		1.0	0.97	-3
		2.0	2.00	0
		4.0	3.93	-2
		8.0	7.93	-1
		16.0	15.9	-1
	5 August 1985	0.5	0.487	-3
		1.0	1.01	+1
		2.0	2.00	0
		4.0	4.22	+6
		8.0	8.16	+2
		16.0	16.1	+1

^a The dosing volume was adjusted weekly following mean body weight measurements, but did not exceed 300 μ L for rats or 100 μ L for mice. For rats, 1.563 mg/mL=1.563 mg/kg; 3.125 mg/mL=3.125 mg/kg; 6.25 mg/mL=6.25 mg/kg; 12.5 mg/mL=12.5 mg/kg; 25 mg/mL=25 mg/kg. For mice, 0.5 mg/mL=1.563 mg/kg; 1 mg/mL=3.125 mg/kg; 2 mg/mL=6.25 mg/kg; 4 mg/mL=12.5 mg/kg; 8 mg/mL=25 mg/kg.

^b Results of duplicate analyses

^c Animal room samples

^d Result is average of analysis of three samples

^e Because of an interfering static charge on the prep balance, samples originally mixed on 6 June 1985 were remixed and reanalyzed on 10 June 1985.

TABLE G4
Results of Analysis of Dose Formulations Administered to Rats and Mice
in the 2-Year Dermal Studies of Benzethonium Chloride (continued)

Date Prepared	Date Analyzed	Target Concentration (mg/mL)	Determined Concentration (mg/mL)	% Difference from Target
Rats (continued)				
16 March	17 March 1988	0.15	0.146	-3
		0.25	0.250	0
		0.5	0.489	-2
		0.83	0.837	+1
		1.5	1.53	+2
		2.5	2.53	+1
11 May 1988	12 May 1988	0.15	0.150	0
		0.25	0.249	0
		0.5	0.499	0
		0.83	0.837	+1
		1.5	1.54	+3
		2.5	2.60	+4
	26 May 1988 ^d	0.15	0.155	+3
		0.25	0.255	+2
		0.5	0.502	0
		0.83	0.857	+3
		1.5	1.52	+1
		2.5	2.54	+2
5 July 1988	6 July 1988	0.15	0.146	-3
		0.25	0.243	-3
		0.5	0.487	-3
		0.83	0.807	-3
		1.5	1.47	-2
		2.5	2.44	-2
30 August 1988	31 August 1988	0.15	0.148	+1
		0.25	0.246	-2
		0.5	0.490	-2
		0.83	0.815	-2
		1.5	1.49	-1
		2.5	2.47	-1
26 September 1988	28 September 1988	0.15	0.149	-1
		0.25	0.251	0
		0.5	0.500	0
		0.83	0.825	-1
		1.5	1.54	+3
		2.5	2.52	+1
	14 November 1988 ^d	0.15	0.161	+7
		0.25	0.261	+4
		0.5	0.509	+2
		0.83	0.850	+2
		1.5	1.55	+3
		2.5	2.54	+2

TABLE G4
Results of Analysis of Dose Formulations Administered to Rats and Mice
in the 2-Year Dermal Studies of Benzethonium Chloride (continued)

Date Prepared	Date Analyzed	Target Concentration (mg/mL)	Determined Concentration (mg/mL)	% Difference from Target
Rats (continued)				
27 December 1988	29 December 1988	0.15	0.148	-1
		0.25	0.246	-2
		0.5	0.504	+1
		0.83	0.823	-1
		1.5	1.50	0
		2.5	2.48	-1
22 February 1989	23 February 1989	0.15	0.157	+5
		0.25	0.267	+7
		0.5	0.525	+5
		0.83	0.877	+6
		1.5	1.52	+1
		2.5	2.71	+8
19 April 1989	20 April 1989	0.15	0.149	-1
		0.25	0.246	-2
		0.5	0.493	-1
		0.83	0.820	-1
		1.5	1.49	-1
		2.5	2.47	-1
	2 May 1989 ^d	0.15	0.157	+5
		0.25	0.249	0
		0.5	0.505	+1
		0.83	0.837	+1
		1.5	1.51	+1
		2.5	2.49	0
Mice				
15 June 1987	15 June 1987	0.06	0.060 ^c	0
		0.2	0.196 ^c	-2
		0.6	0.595	-1
	29 June 1987 ^d	0.06	0.0658	+10
		0.2	0.204	+2
		0.6	0.613	+2
5 August 1987	6 August 1987	0.06	0.0574 ^c	-4
		0.2	0.198 ^c	-1
		0.6	0.604	+1
30 September 1987	1 October 1987	0.06	0.0561	-6
		0.2	0.195	-2
		0.6	0.603	+1

TABLE G4
Results of Analysis of Dose Formulations Administered to Rats and Mice
in the 2-Year Dermal Studies of Benzethonium Chloride (continued)

Date Prepared	Date Analyzed	Target Concentration (mg/mL)	Determined Concentration (mg/mL)	% Difference from Target
Mice (continued)				
9 December 1987	10 December 1987	0.06	0.055	-8
		0.2	0.198	-1
		0.6	0.589	-2
	4 January 1988 ^d	0.06	0.056	-7
		0.2	0.211	+6
		0.6	0.627	+5
20 January 1988	21 January 1988	0.06	0.058	-3
		0.2	0.198	-1
		0.6	0.594	-1
16 March 1988	17 March 1988	0.06	0.058	-3
		0.2	0.193	-3
		0.6	0.582	+3
11 May 1988	12 May 1988	0.06	0.059	-2
		0.2	0.201	+1
		0.6	0.599	0
	26 May 1988 ^d	0.06	0.059	-2
		0.2	0.204	+2
		0.6	0.616	+3
5 July 1988	6 July 1988	0.06	0.058	-3
		0.2	0.194	-3
		0.6	0.585	-2
30 August 1988	31 August 1988	0.06	0.061	+2
		0.2	0.197	-1
		0.6	0.585	-2
26 October 1988	28 October 1988	0.06	0.063	+5
		0.2	0.201	+1
		0.6	0.597	0
	14 November 1988 ^d	0.06	0.063	+5
		0.2	0.203	+2
		0.6	0.605	+1
27 December 1988	29 December 1988	0.06	0.059	-2
		0.2	0.196	-2
		0.6	0.590	-2
22 February 1988	23 February 1988	0.06	0.062	+3
		0.2	0.210	+5
		0.6	0.623	+4

TABLE G4
Results of Analysis of Dose Formulations Administered to Rats and Mice
in the 2-Year Dermal Studies of Benzethonium Chloride (continued)

Date Prepared	Date Analyzed	Target Concentration (mg/mL)	Determined Concentration (mg/mL)	% Difference from Target
Mice (continued)				
19 April 1988	20 April 1988	0.06	0.058	-3
		0.2	0.198	-1
		0.6	0.585	-2
	2 May 1989	0.06	0.073	+22
		0.2	0.206	+3
		0.6	0.619	+3

^a The dosing volume was based on mean body weight measurements and ranged from 63-296 μ L for male rats, 95-317 μ L for female rats, and 50-131 μ L for male and female mice. For male rats, 0.25 mg/mL=0.15 mg/kg, 0.83 mg/mL=0.5 mg/kg, 2.5 mg/mL=1.5 mg/kg. For female rats, 0.15 mg/mL=0.15 mg/kg, 0.5 mg/mL=0.5 mg/kg, 1.5 mg/mL=1.5 mg/kg. For mice, 0.06 mg/mL=0.15 mg/kg, 0.2 mg/mL=0.5 mg/kg, 0.6 mg/mL=1.5 mg/kg.

^b Results of duplicate analyses

^c Results of single analysis

^d Animal room sample

TABLE G5
Results of Referee Analysis of Dose Formulations Administered to Rats and Mice
in the 2-Year Dermal Studies of Benzethonium Chloride

Date Mixed	Target Concentration (mg/mL)	Determined Concentration (mg/mL)	
		Study Laboratory ^a	Referee Laboratory ^b
13 Weeks			
25 April 1985	1.0	0.96	1.04 ± 0.01
18 July 1985	8.0	7.93	7.99 ± 0.01
2 Years			
Rats			
9 June 1987	0.25	0.249	1.12 ± 0.0 ^c
	0.83	0.829	0.830 ± 0.002
11 May 1988	2.5	2.60	2.51 ± 0.01
19 April 1989	0.83	0.820	0.922 ± 0.003
Mice			
9 December 1987	0.2	0.198	0.193 ± 0.008
26 October 1988	0.06	0.063	0.0583 ± 0.0002

^a Results of duplicate analyses

^b Results of triplicate analyses

^c No explanation for this discrepancy was identified.

APPENDIX H
INGREDIENTS, NUTRIENT COMPOSITION,
AND CONTAMINANT LEVELS
IN NIH-07 RAT AND MOUSE RATION

TABLE H1	Ingredients of NIH-07 Rat and Mouse Ration	H-2
TABLE H2	Vitamins and Minerals in NIH-07 Rat and Mouse Ration	H-2
TABLE H3	Nutrient Composition of NIH-07 Rat and Mouse Ration	H-3
TABLE H4	Contaminant Levels in NIH-07 Rat and Mouse Ration	H-4

TABLE H1
Ingredients of NIH-07 Rat and Mouse Ration^a

Ingredients ^b	Percent by Weight
Ground #2 yellow shelled corn	24.50
Ground hard winter wheat	23.00
Soybean meal (49% protein)	12.00
Fish meal (60% protein)	10.00
Wheat middlings	10.00
Dried skim milk	5.00
Alfalfa meal (dehydrated, 17% protein)	4.00
Corn gluten meal (60% protein)	3.00
Soy oil	2.50
Dried brewer's yeast	2.00
Dry molasses	1.50
Dicalcium phosphate	1.25
Ground limestone	0.50
Salt	0.50
Premixes (vitamin and mineral)	0.25

^a NCI, 1976; NIH, 1978

^b Ingredients were ground to pass through a U.S. Standard Screen No. 16 before being mixed.

TABLE H2
Vitamins and Minerals in NIH-07 Rat and Mouse Ration^a

	Amount	Source
Vitamins		
A	5,500,000 IU	Stabilized vitamin A palmitate or acetate
D ₃	4,600,000 IU	D-activated animal sterol
K ₃	2.8 g	Menadione
<i>d</i> - α -Tocopheryl acetate	20,000 IU	
Choline	560.0 g	Choline chloride
Folic acid	2.2 g	
Niacin	30.0 g	
<i>d</i> -Pantothenic acid	18.0 g	<i>d</i> -Calcium pantothenate
Riboflavin	3.4 g	
Thiamine	10.0 g	Thiamine mononitrate
B ₁₂	4,000 μ g	
Pyridoxine	1.7 g	Pyridoxine hydrochloride
Biotin	140.0 mg	<i>d</i> -Biotin
Minerals		
Iron	120.0 g	Iron sulfate
Manganese	60.0 g	Manganous oxide
Zinc	16.0 g	Zinc oxide
Copper	4.0 g	Copper sulfate
Iodine	1.4 g	Calcium iodate
Cobalt	0.4 g	Cobalt carbonate

^a Per ton (2,000 lb) of finished product

TABLE H3
Nutrient Composition of NIH-07 Rat and Mouse Ration

Nutrient	Mean \pm Standard Deviation	Range	Number of Samples
Protein (% by weight)	22.76 \pm 0.79	21.70 – 24.20	25
Crude Fat (% by weight)	5.39 \pm 0.37	4.60 – 5.90	25
Crude Fiber (% by weight)	3.52 \pm 0.31	2.80 – 4.20	25
Ash (% by weight)	6.81 \pm 0.25	6.26 – 7.30	25
Amino Acids (% of total diet)			
Arginine	1.287 \pm 0.084	1.100 – 1.390	10
Cystine	0.306 \pm 0.075	0.181 – 0.400	10
Glycine	1.160 \pm 0.050	1.060 – 1.220	10
Histidine	0.580 \pm 0.024	0.531 – 0.608	10
Isoleucine	0.917 \pm 0.034	0.867 – 0.965	10
Leucine	1.972 \pm 0.052	1.850 – 2.040	10
Lysine	1.273 \pm 0.051	1.200 – 1.370	10
Methionine	0.437 \pm 0.115	0.306 – 0.699	10
Phenylalanine	0.994 \pm 0.125	0.665 – 1.110	10
Threonine	0.896 \pm 0.055	0.824 – 0.985	10
Tryptophan	0.223 \pm 0.160	0.107 – 0.671	10
Tyrosine	0.677 \pm 0.105	0.564 – 0.794	10
Valine	1.089 \pm 0.057	0.962 – 1.170	10
Essential Fatty Acids (% of total diet)			
Linoleic	2.389 \pm 0.233	1.830 – 2.570	9
Linolenic	0.277 \pm 0.036	0.210 – 0.320	9
Vitamins			
Vitamin A (IU/kg)	6,750 \pm 1,439	4,430 – 10,860	25
Vitamin D (IU/kg)	4,450 \pm 1,382	3,000 – 6,300	4
α -Tocopherol (ppm)	36.92 \pm 9.32	22.5 – 48.9	9
Thiamine (ppm)	18.64 \pm 2.12	14.0 – 23.0	25
Riboflavin (ppm)	7.92 \pm 0.93	6.10 – 9.00	10
Niacin (ppm)	100.95 \pm 25.92	65.0 – 150.0	9
Pantothenic Acid (ppm)	30.30 \pm 3.60	23.0 – 34.6	10
Pyridoxine (ppm)	9.25 \pm 2.62	5.60 – 14.0	10
Folic Acid (ppm)	2.51 \pm 0.64	1.80 – 3.70	10
Biotin (ppm)	0.267 \pm 0.049	0.19 – 0.35	10
Vitamin B ₁₂ (ppb)	40.14 \pm 20.04	10.6 – 65.0	10
Choline (ppm)	3,068 \pm 314	2,400 – 3,430	9
Minerals			
Calcium (%)	1.29 \pm 0.12	1.00 – 1.54	25
Phosphorus (%)	0.95 \pm 0.04	0.86 – 1.00	25
Potassium (%)	0.887 \pm 0.067	0.772 – 0.971	8
Chloride (%)	0.526 \pm 0.092	0.380 – 0.635	8
Sodium (%)	0.315 \pm 0.344	0.258 – 0.370	10
Magnesium (%)	0.168 \pm 0.008	0.151 – 0.180	10
Sulfur (%)	0.274 \pm 0.063	0.208 – 0.420	10
Iron (ppm)	356.2 \pm 90.0	255.0 – 523.0	10
Manganese (ppm)	92.24 \pm 5.35	81.70 – 99.40	10
Zinc (ppm)	58.14 \pm 9.91	46.10 – 81.60	10
Copper (ppm)	11.50 \pm 2.40	8.090 – 15.39	10
Iodine (ppm)	3.70 \pm 1.14	1.52 – 5.83	10
Chromium (ppm)	1.71 \pm 0.45	0.85 – 2.09	9
Cobalt (ppm)	0.797 \pm 0.23	0.490 – 1.150	6

TABLE H4
Contaminant Levels in NIH-07 Rat and Mouse Ration

	Mean \pm Standard Deviation ^a	Range	Number of Samples
Contaminants			
Arsenic (ppm)	0.18 \pm 0.12	0.05 – 0.55	25
Cadmium (ppm)	0.10 \pm 0.02	<0.10 – 0.20	25
Lead (ppm)	0.32 \pm 0.26	0.05 – 1.00	25
Mercury (ppm)	0.05 \pm 0.01	0.05 – 0.11	25
Selenium (ppm) ^b	0.39 \pm 0.20	0.16 – 1.21	25
Aflatoxins (ppb)	<5.0		25
Nitrate nitrogen (ppm) ^c	20.04 \pm 7.55	9.90 – 39.0	25
Nitrite nitrogen (ppm) ^c	0.19 \pm 0.14	<0.10 – 0.60	25
BHA (ppm) ^d	1.88 \pm 0.51	<0.10 – 3.00	25
BHT (ppm) ^d	1.12 \pm 0.52	<0.10 – 3.00	25
Aerobic plate count (CFU/g) ^{e,f}	132,320 \pm 192,307	13,000 – 940,000	25
Coliform (MPN/g) ^g	55.60 \pm 219.57	3.00 – 11.0	25
<i>E. coli</i> (MPN/g)	3.04 \pm 0.20	3.00 – 4.00	25
<i>Salmonella</i> (MPN/g)	Negative		25
Total Nitrosoamines (ppb) ^h	10.65 \pm 4.99	3.60 – 20.00	25
<i>N</i> -Nitrosodimethylamine (ppb)	8.30 \pm 4.62	2.60 – 19.00	25
<i>N</i> -Nitrosopyrrolidine (ppb)	2.36 \pm 1.43	0.90 – 5.40	25
Pesticides (ppm)			
α -BHC ⁱ	<0.01		25
β -BHC	<0.02		25
γ -BHC	<0.01		25
δ -BHC	<0.01		25
Heptachlor	<0.01		25
Aldrin	<0.01		25
Heptachlor epoxide	<0.01		25
DDE	<0.01		25
DDD	<0.01		25
DDT	<0.01		25
HCB	<0.01		25
Mirex	<0.01		25
Methoxychlor	<0.05		25
Dieldrin	<0.01		25
Endrin	<0.01		25
Telodrin	<0.01		25
Chlordane	<0.05		25
Toxaphene	<0.1		25
Estimated PCBs	<0.2		25
Ronnel	<0.01		25
Ethion	<0.02		25
Trithion	<0.05		25
Diazinon	<0.1		25
Methyl parathion	<0.02		25
Ethyl parathion	<0.02		25
Malathion	0.19 \pm 0.17	<0.05 – 0.60	25
Endosulfan I	<0.01		25
Endosulfan II	<0.01		25
Endosulfan sulfate	<0.03		25

TABLE H4
Contaminant Levels in NIH-07 Rat and Mouse Ration (continued)

- ^a For values less than the limit of detection, the detection limit is given as the mean.
- ^b One lot milled on 2 March 1989 contained more than 0.65 ppm. All other lots measured less than or equal to the detection limit.
- ^c Sources of contamination: alfalfa, grains, and fish meal
- ^d Sources of contamination: soy oil and fish meal
- ^e CFU = colony forming unit
- ^f One lot milled on 5 November 1987 contained more than 600,000 CFU/g.
- ^g MPN = most probable number
- ^h All values were corrected for percent recovery.
- ⁱ BHC is hexachlorocyclohexane or benzene hexachloride

APPENDIX I
SENTINEL ANIMAL PROGRAM

METHODS **I-2**
TABLE II **Murine Virus Antibody Determinations for Rats and Mice**
 in the 13-Week and 2-Year Dermal Studies of Benzethonium Chloride **I-4**

SENTINEL ANIMAL PROGRAM

METHODS

Rodents used in the Carcinogenesis Program of the National Toxicology Program are produced in optimally clean facilities to eliminate potential pathogens that may affect study results. The Sentinel Animal Program is part of the periodic monitoring of animal health that occurs during the toxicologic evaluation of chemical compounds. Under this program, the disease state of the rodents is monitored via serology on sera from extra (sentinel) animals in the study rooms. These animals and the study animals are subject to identical environmental conditions. The sentinel animals come from the same production source and weanling groups as the animals used for the studies of chemical compounds.

Rats

At the end of the 13-week study, samples for viral screening were collected from five male and five female vehicle control rats. These samples were processed appropriately and submitted to Microbiological Associates, Inc. (Bethesda, MD), for viral titer screening. The following tests were performed on the sera:

Method of Analysis

Time of Analysis

ELISA

Mycoplasma arthritidis

Study termination

Mycoplasma pulmonis

Study termination

PVM (pneumonia virus of mice)

Study termination

RCV/SDA (rat coronavirus/sialodacryoadenitis virus)

Study termination

Sendai

Study termination

Hemagglutination Inhibition

H-1 (Toolan's H-1 virus)

Study termination

KRV (Kilham rat virus)

Study termination

Prior to the beginning of the 2-year study, blood was collected once (during one quarantine screening) from five male and five female rats. Serum samples were also collected from five male and five female rats at 6, 12, and 18 months into the study and from five male and five female high-dose rats at the end of the study (24 months). Blood from each collection was processed appropriately, shipped to Microbiological Associates, Inc., and screened for the following:

Method of Analysis

Time of Analysis

ELISA

M. arthritidis

24 months

M. pulmonis

24 months

PVM

Quarantine, 6, 12, 18, and 24 months

RCV/SDA

Quarantine, 6, 12, 18, and 24 months

Sendai

Quarantine, 6, 12, 18, and 24 months

Hemagglutination Inhibition

H-1

Quarantine, 6, 12, 18, and 24 months

KRV

Quarantine, 6, 12, 18, and 24 months

Mice

At the end of the 13-week study, samples for viral screening were collected from five male and five female vehicle control mice. These samples were processed appropriately and submitted to Microbiological Associates, Inc., for viral titer screening. The following tests were performed on the sera:

<u>Method of Analysis</u>	<u>Time of Analysis</u>
Complement Fixation	
LCM (lymphocytic choriomeningitis virus)	Study termination
ELISA	
Ectromelia virus	Study termination
GDVII (mouse encephalomyelitis virus)	Study termination
Mouse adenoma virus	Study termination
MHV (mouse hepatitis virus)	Study termination
<i>M. arthritidis</i>	Study termination
<i>M. pulmonis</i>	Study termination
PVM	Study termination
Reovirus 3	Study termination
Sendai	Study termination
Hemagglutination Inhibition	
K (papovavirus)	Study termination
MVM (minute virus of mice)	Study termination
Polyoma virus	Study termination
Immunofluorescence Assay	
EDIM (epizootic diarrhea of infant mice)	Study termination

Prior to the beginning of the 2-year study, blood was collected once (during one quarantine screening) from five male and five female mice. Serum samples were also collected from five males and five females at 6, 12, and 18 months into the study and from five male and five female high-dose mice at the end of the study (24 months). In addition, an unscheduled screening was conducted on five male and five female vehicle control mice at about 22 months into the study. Blood from each collection was processed appropriately, shipped to Microbiological Associates, Inc., and screened for the following:

<u>Method of Analysis</u>	<u>Time of Analysis</u>
ELISA	
Ectromelia virus	Quarantine, 6, 12, 18, 22, and 24 months
GDVII	Quarantine, 6, 12, 18, 22, and 24 months
LCM	Quarantine, 6, and 12 months
MVM	Quarantine, 6, 12, 18, 22, and 24 months
Mouse adenoma virus	Quarantine, 6, 12, 18, 22, and 24 months
MHV	Quarantine, 6, 12, 18, 22, and 24 months
<i>M. arthritidis</i>	24 months
<i>M. pulmonis</i>	24 months
PVM	Quarantine, 6, 12, 18, 22, and 24 months
Reovirus 3	Quarantine, 6, 18, 22, and 24 months
Sendai	Quarantine, 6, 12, 18, 22, and 24 months
Hemagglutination Inhibition	
K	Quarantine, 6, 12, 18, 22, and 24 months
Polyoma virus	Quarantine, 6, 12, 18, 22, and 24 months

Mice (continued)Method of Analysis

Immunofluorescence Assay

EDIM

LCM

Reovirus 3

Time of Analysis

Quarantine, 6, 12, 18, 22, and 24 months

18, 22, and 24 months

12 months

Serology results are presented in Table I1.

TABLE I1**Murine Virus Antibody Determinations for Rats and Mice in the 13-Week and 2-Year Dermal Studies of Benzethonium Chloride**

Interval	Incidence of Antibody in Sentinel Animals	Positive Serologic Reaction for
13-Week Studies		
Rats		
Study termination	1/10	<i>M. arthritidis</i> ^a
Mice		
Study termination	0/10	None positive
2-Year Studies		
Rats		
Quarantine screening	0/10	None positive
6 months	0/10	None positive
12 months	0/10	None positive
18 months	0/10	None positive
24 months	2/10	<i>M. arthritidis</i> ^a
Mice		
Quarantine screening	0/10	None positive
6 months	0/10	None positive
12 months	0/10	None positive
18 months	0/10	None positive
22 months	7/10	Mouse hepatitis virus
24 months	8/10	Mouse hepatitis virus

^a Possible *Mycoplasma arthritidis*

ABSTRACT

A subchronic dermal toxicity study of benzethonium chloride was conducted in young adult Fischer 344 rats. The test chemical, formulated in ethanol, was applied to the dorsal skin of rats (10/sex/group) at dose levels of 25, 12.5, 6.25, 3.125, 1.563, or 0 mg/kg, five times per week for 13 weeks.

No unscheduled deaths occurred in this study. A treatment-related reduction in body weight occurred in male rats exposed to 25 mg/kg of benzethonium chloride. No other body weight differences between the treatment and control groups of either sex were observed. The incidence of skin irritation in the dosed-skin region and the time that lesions first appeared were both treatment- and dose-related. Treatment-associated gross changes observed at necropsy were limited to the dosed skin area of all treatment groups. Multiple, irregular, epidermal crusts and thickened skin occurred frequently in rats treated with 25 mg/kg or 12.5 mg/kg of the test material with multiple red foci observed in rats of each dose level. Dosed skin was the only target organ identified by microscopic examination. All rats in the high dose group had necrotizing inflammation, with the incidence and severity of this lesion decreasing through the lower dose groups. Additionally, chronic dermatitis, hyperkeratosis, and acanthosis were present in treated rats of all but the high dose group. A summary of the toxicology results of this study is presented in Table 1.

TABLE 1. SUMMARY OF TOXICOLOGIC DATA FOR RATS ON THE SUBCHRONIC DERMAL STUDY OF BENZETHONIUM CHLORIDE IN FISCHER 344 RATS

Dose Level, mg/kg	Mortality	Mean Body Weight Gain, grams (Percent Change Relative to Control ^a)	Clinical Signs	Significant Necropsy Findings	Significant Microscopic Findings
Males					
25	0/10	101.4 (-25.3)	Irritation (10/10) at site of application; nasal discharge (1/10)	Crusts (10/10), thickening (6/10) at site of application	Skin, treated: necrotizing, inflammation (10/10)
12.5	0/10	123.3 (-6.9)	Irritation (10/10) at site of application	Crusts (4/10), thickening (1/10), red foci (2/10), and lesion (1/10) at site of application	Skin, treated: necrotizing inflammation (8/10), chronic inflammation of dermis (1/10), hyperkeratosis (2/10), and acanthosis (1/10)
6.25	0/10	127.5 (-3.8)	Irritation (7/10) at site of application	Crusts (2/10) and red foci (5/10) at site of application	Skin, treated: necrotizing inflammation (6/10), chronic inflammation of dermis (3/10), hyperkeratosis (2/10), and acanthosis (2/10)
3.125	0/10	134.8 (1.7)	Irritation (3/10) and clipper injury (1/10) at site of application	Red foci (4/10) at site of application	Skin, treated: necrotizing inflammation (3/10), chronic inflammation of dermis (2/10), hyperkeratosis (1/10), and acanthosis (2/10)
1.563	0/10	129.0 (-2.6)	None	None	Skin, treated: necrotizing inflammation (1/10)
0	0/10	132.5	Discharge, right eye (1/10)	None	None

TABLE 1. (Continued)

Dose Level, mg/kg	Mortality	Mean Body Weight Gain, grams (Percent Change Relative to Control ^a)	Clinical Signs	Significant Necropsy Findings	Significant Microscopic Findings
<u>Females</u>					
25	0/10	51.2 (-8.9)	Scaly (1/10); irritation (10/10) and discoloration (1/10) at site of application	Crusts (10/10) and thickening (2/10) at site of application	Skin, treated: necrotizing inflammation (10/10)
12.5	0/10	52.3 (-6.9)	Scaly (2/10), and irritation (9/10) at site of application	Crusts (3/10) and red foci (4/10) at site of application	Skin, treated: necrotizing inflammation (7/10), chronic inflammation of dermis (3/10), hyperkeratosis (1/10), and acanthosis (2/10)
6.25	0/10	52.6 (-6.4)	Irritation (7/10) at site of application	Crusts (3/10) and red foci (3/10) at site of application	Skin, treated: necrotizing inflammation (4/10), chronic inflammation of dermis (4/10), hyperkeratosis (1/10), and acanthosis (3/10)
3.125	0/10	52.9 (-5.9)	Irritation (8/10) at site of application	Crusts (2/10) and red foci (4/10) at site of application	Skin, treated: necrotizing inflammation (5/10), chronic inflammation of dermis (4/10), hyperkeratosis (2/10), and acanthosis (3/10)
1.563	0/10	54.5 (-3.0)	Irritation (1/10) at site of application	Red foci (2/10) at site of application	Skin, treated: chronic inflammation of dermis (3/10), and hyperkeratosis (2/10)
0	0/10	56.2	None	None	None

^a Percent Change Relative to Control = $\frac{\text{Mean Value for Dosed Group} - \text{Mean Value for Control Group}}{\text{Mean Value for Control Group}} \times 100$

ABSTRACT

A subchronic study was conducted in which ten B6C3F1 mice of each sex were administered 25, 12.5, 6.25, 3.125, 1.563, or 0 mg/kg benzethonium chloride dermally (five times per week) for 13 weeks. No mortality occurred during this study. Chemical administration produced an initial irritation or redness, crustiness, depigmentation of fur, and thickening of the skin in the dosed area predominately in high dose mice of both sexes. All male dose groups and the 12.5 and 25 mg/kg female dose groups exhibited a depression in differential weight gain relative to their respective control group over the course of the study. This effect was dose-related in males (at the 6.25 mg/kg level and above) but not in the female dose groups. At study termination, no consistent or biologically significant alterations were seen between treated and control mice of either sex in mean organ absolute weights or in the organ weight to body weight or brain weight ratios. Upon histopathological examination, chemically-induced microscopic abnormalities were restricted to the dosed area. Epidermal hyperkeratosis and acanthosis occurred in all benzethonium chloride dose groups of both sexes. Necrotizing epidermal inflammation was restricted to one male mouse at the 12.5 mg/kg dose level and to several mice of both sexes at the 25.0 mg/kg dose level. Chronic dermal inflammation occurred mainly at the 6.25 mg/kg dose level and above in mice of both sexes. A summary of the results of this study is presented in Table 1.

TABLE 1. SUMMARY OF TOXICOLOGIC DATA FOR MICE ON THE DERMAL SUBCHRONIC STUDY OF BENZETHONIUM CHLORIDE IN B6C3F1 MICE

Dose Level, mg/kg	Mortality	Mean Body Weight Gain, grams (Percent Change Relative to Control ^a)	Clinical Signs	Significant Necropsy Findings	Significant Microscopic Findings
<u>Males</u>					
25	0/10	6.8 (-26.1)	Alopecia, body ventral (1/10); discoloration, hair (4/10); prolapse, penis (1/10); scaly (9/10), thickened (5/10), irri- tation (7/10), and discolora- tion (10/10), site of appli- cation	Skin: crust (1/10), and hair, pigment, white (6/10)	Skin, treated: dermis, inflammation, chronic (6/10); epidermis, hyperkeratosis (5/10) and acanthosis (6/10); inflammation, necrotizing (4/10)
12.5	0/10	7.8 (-15.2)	Alopecia, body ventral (1/10); scaly (5/10), thickened (3/10), irritation (1/10), and dis- coloration (5/10), site of application	Skin: crust (1/10), and hair, pigment, white (1/10)	Skin, treated: dermis, inflammation, chronic (8/10); epidermis, hyperkeratosis (8/10) and acanthosis (8/10); inflammation, necrotizing (1/10)
6.25	0/10	7.9 (-14.1)	Alopecia, body ventral (1/10); thickened, site of application (1/10)	None	Skin, treated: dermis, inflammation, chronic (6/10); epidermis, hyperkeratosis (9/10) and acanthosis (10/10)
3.125	0/10	8.5 (-7.6)	Prolapse, penis (1/10)	None	Skin, treated: dermis, inflammation, chronic (1/10); epidermis, hyperkeratosis (6/10) and acanthosis (8/10)
1.563	0/10	8.3 (-9.8)	None	None	Skin, treated: epidermis, hyperkeratosis (8/10) and acanthosis (7/10)
0	0/10	9.2	None	None	None

TABLE 1. (Continued)

Dose Level, mg/kg	Mortality	Mean Body Weight Gain, grams (Percent Change Relative to Control ^a)	Clinical Signs	Significant Necropsy Findings	Significant Microscopic Findings
<u>Females</u>					
25	0/10	7.0 (-7.9)	Alopecia, right rear leg (1/10); alopecia, body ventral (4/10); discoloration, hair (4/10); scaly (6/10), thickened (2/10), irritation (2/10), and discoloration (9/10), site of application	Skin: crust (1/10) and hair, pigment, white (4/10)	Skin, treated: dermis, inflammation, chronic (7/10); epidermis, hyperkeratosis (6/10) and acanthosis (6/10); inflammation, necrotizing (3/10)
12.5	0/10	6.7 (-11.8)	Alopecia, right rear leg (1/10); alopecia, anal area (1/10); alopecia, body ventral (1/10); scaly (2/10) and discoloration (1/10), site of application	Skin: crust (1/10)	Skin, treated: dermis, inflammation, chronic (10/10); epidermis, hyperkeratosis (10/10) and acanthosis (10/10)
6.25	0/10	8.1 (6.6)	None	None	Skin, treated: dermis, inflammation, chronic (7/10); epidermis, hyperkeratosis (10/10) and acanthosis (10/10)
3.125	0/10	7.9 (3.9)	Alopecia, body ventral (1/10)	None	Skin, treated: epidermis, hyperkeratosis (10/10) and acanthosis (10/10)
1.563	0/10	7.7 (1.3)	None	Skin: scar (1/10)	Skin, treated: dermis, inflammation, chronic (2/10); epidermis, hyperkeratosis (9/10) and acanthosis (10/10)
0	0/10	7.6	Clipper injury, site of application (1/10)	None	Skin, treated: dermis, inflammation, chronic (1/10)

^a Percent Change Relative to Control = $\frac{\text{Mean Value for Dosed Group} - \text{Mean Value for Control Group}}{\text{Mean Value for Control Group}} \times 100$

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Pathology Working Group

**Benzethonium Chloride (C61494)
13 Week Subchronic Dermal Toxicity
Study in F344 Rats and B6C3F1 Mice
May 1, 1986**

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NTS DATA UNIT

Conflict of Interest Statement

Those individuals involved in testing or evaluation of this chemical (Benzethonium Chloride, C61494) at Pathology Associates, Inc. are listed below.

Pathology - Michael A. Stedham, DVM, MS
Clerical - Janet M. Bromfield
Terri Smith

They have not been involved in the testing or evaluation of this chemical for clients other than the National Toxicology Program.

Body Weight

The difference in weight gain (%) at 13 weeks compared to the controls is listed by dose group.

<u>Group</u>	<u>Male</u>	<u>Female</u>
1.563	-2.6	-3.0
3.125	+1.7	-5.9
6.25	-3.8	-6.4
12.5	-6.9	-6.9
25.0	-23.5	-8.9

The only large decrease in weight gain was in the 25.0 mg/kg males.

Organ Weight

Absolute organ weights, organ to body weight ratios, and organ to brain weight ratios were performed for thymus, liver, kidney, testis, heart, lung, and brain. As noted in the original report, the majority of significant differences were in the high dose groups (25 mg/kg) of male rats. The only significant difference in absolute organ weight was for the thymus (decreased) in the high dose male group.

Clinical Pathology

None performed.

Clinical Signs

"Skin irritation", not further defined, occurred in a dose-related pattern in both males and females as follows:

<u>Group</u>	<u>Male</u> <u>n=10</u>	<u>Female</u> <u>n=10</u>
0.0	0	0
1.563	0	1
3.125	3	8
6.25	7	7
12.5	10	9
25.0	10	10

Scaliness or discoloration was reported in a few females in the two highest dose groups.

Necropsy Data

As noted in the original report, multiple irregular, epidermal crusts and thickened skin were the predominant lesions, the highest frequency occurring in the 25.0 and 12.5 mg/kg groups. Red foci were noted in rats in the other dose groups.

Histopathology

The original pathologist (OP) recorded inflammatory (including necrotizing) and lesions of the skin in all dose groups, and proliferative lesions in all dose groups but the highest one. The inflammatory lesions increased in incidence and severity with increased doses. The OP, however, failed to record the proliferative lesions (acanthosis, hyperkeratosis) in the high dose groups (25 mg/kg, male and female) when he rendered a diagnosis of necrotizing inflammation, thus making proper interpretation of the original table impossible with regard to these lesions. As pointed out by the QAP, these lesions were, in fact, present in the 25 mg/kg groups and they were more severe than in the other groups.

The QAP correctly points out that "a thorough description of the lesions noted should have been in the histopathology narrative describing the spectrum of lesions and the sequential reduction in severity and incidences with decreasing dosage." The QAP defines necrotizing inflammation for the purposes of his review as encompassing acanthosis, hyperkeratosis, and chronic inflammation as well. This may have been the intent of the OP as well but it is not stated.

In the QAP comments (diagnoses) column for the most part, additional diagnoses for proliferative epithelial changes are not rendered when he has concurred with the OP diagnosis of necrotizing inflammation. An exception to this format is noted in 25 mg/kg female rat #116, Histology #856356.

Both the OP and QAP apparently considered the bone marrow to be normal where as the PWG chairperson considered that myeloid hyperplasia was present in the high dose (25 mg/kg) male and female rats. Bones from the other dosed groups were not available for review because marrow had not been detected as a target organ. It is suggested that the hyperplasia is

secondary to the inflammatory skin lesions and is not a primary target. The full PWG preferred the term, hypercellularity, for the marrow lesion but concurred that a difference was present between the control and 25 mg/kg rats.

Other differences in diagnoses between the OP, QAP, and the PWG chairman were minor, were related to terminology, or were in non-target tissues. In the last category was inflammation of the nasal mucosa and a fungal colony in the nasal cavity (lumen) of Animal No. 56 in the 25 mg/kg male group.

A rudimentary description of the lesions is offered. In general, all components of the inflammatory and proliferative lesions decreased in incidence and severity with decreasing dose and were roughly equal between sexes.

All components of the epidermis of treated skin proliferated to some degree in many of the more severely affected animals in the higher dose groups. Acanthosis was the most prominent feature, generally, followed by hyperkeratosis and lesser degrees of basal cell hyperplasia or alteration, and an increased stratum granulosum. Parakeratosis was also present as was hyperplasia of the sebaceous glands. These were also considered under hyperplasia, epithelium as diagnosed by the PWG Chairperson.

Inflammation, necrotizing included either necrosis or ulceration (presuming previous necrosis) or both of the epidermis plus varying amounts of chronic and acute inflammatory cells in the dermis. In more severe instances the full depth of the dermis was affected with some involvement of the adjacent subcutis. In addition, some degree of collagen degeneration and/or early fibrosis was noted in some areas. In some instances immature fibroblasts and collagen were oriented with their long axis parallel to each other and to the skin surface (granulation tissue). Also, included with the diagnosis of inflammation was accumulation of neutrophils in the epidermis, primarily between the spiny cell layer and the keratin layer (pustules). The epidermal necrosis was coagulative in nature and was frequently full-depth. In some instances, it was difficult to differentiate between parakeratosis and necrosis. In these instances, the epidermis (progressing through spiny cells, granular cells, and keratin) underlaid plumper, poorly staining "ghost"

cells. In addition, occasional accumulations of neutrophils were noted beneath these overlaying "ghost" cells. This may reflect previous full depth necrosis of the epithelium with neutrophil infiltrate and subsequent regeneration of the epithelium from beneath (residual basal cells or follicular epithelium).

When necrosis or ulceration was not present, inflammation, chronic or chronic, active was rendered. Chronic or chronic, active was also used to modify necrotizing inflammation. Although these differences (chronic versus chronic, active) may be legitimate, they merely reflect the presence or absence of neutrophils in a basically chronic reaction, and for purposes of generating more understandable tabular results, could be consolidated under chronic.

Although the QAP apparently attempted to follow the OPs method of diagnosis, the PWG Chairperson preferred to combine the proliferative lesions into hyperplasia, epithelial and to render this diagnosis even if necrotizing inflammation was also diagnosed. This was done in an attempt to simplify tabular data inasmuch as the OP tables were misleading in this regard. Also, it would be more consistent with diagnoses rendered for the mice in the study. Further, although the QAP and the PWG Chairperson had minor differences in diagnosing the proliferative lesions in the treated skin of the lower dose groups, they were in general concurrence that these lesions were undercalled by the OP in these dose groups.

Summary

The PWG considers the 1.56 mg/kg dose level to be appropriate for the chronic study. Higher dose levels were considered to have risk for a chronic study owing to necrosis, ulcers, and inflammation.

The PWG considered the pathology narrative to be unsatisfactory in that the lesions were not characterized and that the tables do not help substantially in clarifying the lesions. In particular, the tables are misleading in reference to hyperplastic/proliferative lesions. Bone marrow should also be considered a target organ, although probably a secondary target.

Action Items

1. The PWG Chairperson will review again the lesions in the treated skin, classify them under chronic inflammation, necrosis, ulcer, and epithelial hyperplasia, and produce tables useful for dose-setting for the chronic study. The PWG felt that it was important to list necrosis and ulcer separately as these may be life-threatening in a chronic study.

2. The slides will be returned to the OP for reconsideration of the sections of treated skin and of bone marrow.

Action Taken

The following summary tables and individual tables from re-evaluation of treated skin by the PWG Chairperson are provided. It should be noted that the full PWG generally evaluated the lesions to have a slightly lower degree of severity (0 to 1 degree lower) than the Chairperson.

SUMMARY TABLE

FEMALE RATS

mg/kg	0.0	1.56	3.12	6.25	12.5	25.0
Inflammation, Chronic	0/10* 0.0**	4/10 1.0	10/10 1.5	7/10 1.6	10/10 1.7	10/10 3.2
Necrosis	0/10 0.0	0/10 0.0	1/10 1.0	3/10 1.3	5/10 1.0	8/10 1.4
Ulcer	0/10 0.0	0/10 0.0	5/10 1.8	3/10 1.7	1/10 1.0	10/10 2.0
Hyperplasia, Epithelial	0/10 0.0	5/10 1.0	9/10 1.4	9/10 1.6	10/10 1.9	10/10 3.0

SUMMARY TABLE

MALE RATS

mg/kg	0.0	1.56	3.12	6.25	12.5	25.0
Inflammation, Chronic	0/10* 0.0**	2/10 1.0	7/10 1.1	9/10 1.7	10/10 2.5	10/10 3.5
Necrosis	0/10 0.0	0/10 0.0	1/10 1.0	2/10 1.0	6/10 1.2	9/10 1.9
Ulcer	0/10 0.0	0/10 0.0	2/10 1.0	4/10 1.5	8/10 1.9	10/10 2.5
Hyperplasia, Epithelial	0/10 0.0	4/10 1.0	9/10 1.1	10/10 1.7	10/10 2.6	10/10 3.0

- * No. affected/No. in group
- ** Average degree of severity
- 0-Within Normal Limits
- 1-Minimal
- 2-Mild
- 3-Moderate
- 4-Marked

**INDIVIDUAL ANIMAL TABLE
MALE RAT**

Control

ANIMAL #	1	2	3	4	5	6	7	8	9	10
Inflammation, chronic	0	0	0	0	0	0	0	0	0	0
Necrosis	0	0	0	0	0	0	0	0	0	0
Ulcer	0	0	0	0	0	0	0	0	0	0
Hyperplasia, Epithelium	0	0	0	0	0	0	0	0	0	0

1.56 mg/kg

ANIMAL #	11	12	13	14	15	16	17	18	19	20
Inflammation, chronic	0	0	1	0	0	0	0	0	0	1
Necrosis	0	0	0	0	0	0	0	0	0	0
Ulcer	0	0	0	0	0	0	0	0	0	0
Hyperplasia, Epithelium	0	1	1	0	0	0	1	0	0	1

3.12 mg/kg

ANIMAL #	21	22	23	24	25	26	27	28	29	30
Inflammation, chronic	1	1	2	1	1	1	0	0	0	1
Necrosis	0	0	1	0	0	0	0	0	0	0
Ulcer	0	0	1	0	0	1	0	0	0	0
Hyperplasia, Epithelium	1	1	1	1	1	2	0	1	1	1

*Average degree of severity
 0-Within Normal Limits
 1-Minimal
 2-Mild
 3-Moderate
 4-Marked

6.25 mg/kg

ANIMAL #	31	32	33	34	35	36	37	38	39	40
Inflammation, chronic	0	1	2	2	1	1	2	2	2	2
Necrosis	0	0	1	1	0	0	0	0	0	0
Ulcer	0	0	0	1	0	0	2	1	0	2
Hyperplasia, Epithelium	1	1	2	2	1	1	2	3	2	2

12.5 mg/kg

ANIMAL #	41	42	43	44	45	46	47	48	49	50
Inflammation, chronic	3	1	4	1	3	3	2	2	2	4
Necrosis	0	0	1	0	2	1	1	1	1	0
Ulcer	1	0	3	0	3	2	1	1	1	3
Hyperplasia, Epithelium	3	2	3	2	3	3	2	3	2	3

25.0 mg/kg

ANIMAL #	51	52	53	54	55	56	57	58	59	60
Inflammation, chronic	3	4	4	4	4	4	3	3	4	2
Necrosis	1	3	2	2	2	2	1	2	2	0
Ulcer	2	2	3	3	3	3	3	2	3	1
Hyperplasia, Epithelium	4	2	3	3	3	3	2	3	4	3

*Average degree of severity

0-Within Normal Limits

1-Minimal

2-Mild

3-Moderate

4-Marked

**INDIVIDUAL ANIMAL TABLE
FEMALE**

Control

ANIMAL #	61	62	63	64	65	66	67	68	69	70
Inflammation, chronic	0	0	0	0	0	0	0	0	0	0
Necrosis	0	0	0	0	0	0	0	0	0	0
Ulcer	0	0	0	0	0	0	0	0	0	0
Hyperplasia, Epithelium	0	0	0	0	0	0	0	0	0	0

1.56 mg/kg

ANIMAL #	71	72	73	74	75	76	77	78	79	80
Inflammation, chronic	1	1	0	1	0	0	1	0	0	0
Necrosis	0	0	0	0	0	0	0	0	0	0
Ulcer	0	0	0	0	0	0	0	0	0	0
Hyperplasia, Epithelium	1	1	0	1	0	0	1	0	0	1

3.12 mg/kg

ANIMAL #	81	82	83	84	85	86	87	88	89	90
Inflammation, chronic	2	1	2	2	1	2	1	1	2	1
Necrosis	0	0	1	0	0	0	0	0	0	0
Ulcer	2	0	0	2	0	2	1	0	2	0
Hyperplasia, Epithelium	2	1	2	2	0	1	1	1	2	1

*Average degree of severity
0-Within Normal Limits
1-Minimal
2-Mild
3-Moderate
4-Marked

6.25 mg/kg

ANIMAL #	91	92	93	94	95	96	97	98	99	100
Inflammation, chronic	0	1	0	3	2	1	0	1	1	2
Necrosis	0	1	0	1	2	0	0	0	0	0
Ulcer	0	0	0	1	2	0	0	0	0	2
Hyperplasia, Epithelium	1	2	0	3	2	1	1	1	1	2

12.5 mg/kg

ANIMAL #	101	102	103	104	105	106	107	108	109	110
Inflammation, chronic	2	1	1	2	2	2	2	2	2	1
Necrosis	0	0	0	1	0	1	1	1	1	0
Ulcer	0	0	0	0	0	0	1	0	0	0
Hyperplasia, Epithelium	2	1	2	2	2	2	2	2	2	2

25.0 mg/kg

ANIMAL #	111	112	113	114	115	116	117	118	119	120
Inflammation, chronic	3	3	3	3	4	4	4	2	3	3
Necrosis	0	1	0	1	2	2	2	1	1	1
Ulcer	1	2	2	2	3	3	3	1	2	1
Hyperplasia, Epithelium	3	3	3	3	3	3	3	3	3	3

*Average degree of severity
 0-Within Normal Limits
 1-Minimal
 2-Mild
 3-Moderate
 4-Marked

B6C3F1 MICE

INTRODUCTION

The study design was:

Dose Level <u>mg/kg</u>	<u>Number of Mice</u>	
	<u>Male</u>	<u>Female</u>
0.0	10	10
1.563	10	10
3.125	10	10
6.25	10	10
12.5	10	10
25.0	10	10

Histotechnique

The QA evaluation of histotechnique was fair to good. The PWG considers it to be acceptable.

As pointed out by the QAP, the location of the skin sites by slide number was not defined in the original report. It was assumed that #2 and #6 were control sites whereas #4 was the treated site with #7, when present, being an additional section of treated skin.

As in the rat study, multiple slides of skin (duplicates or recuts) were present in many animals. These slides usually had no sub-number differentiation. For example, mouse #14 in the 1.56 mg/kg male group had 4 #2s, 2 #4s, 1 #6, and 1 #6A.

Mortality

No deaths or early sacrifices were reported.

Body Weight

The difference in weight gain (%) at 13 weeks as compared to the controls is listed by dose groups.

<u>Group</u>	<u>Male</u>	<u>Female</u>
1.563	-9.8	+1.3
3.125	-7.6	+3.9
6.25	-14.1	+6.6
12.5	-15.2	-11.8
25.0	-26.1	-7.9

In the male groups, the 6.25 mg/kg and higher groups had greater than 10% decrease in weight gain whereas in females only the 12.5 mg/kg group exceeded 10%.

Organ Weight

Absolute organ weights, organ to body weight ratios, and organ to brain weight ratios were performed for the liver, thymus, heart, lungs, right kidney, right testis, and brain. There were no significant differences in absolute organ weights (dosed group vs. control group). The few differences in organ weight relative to body weight occurred primarily in the 12.5 and 25.0 mg/kg male groups which had moderate decreases in weight gain compared to controls.

Clinical Pathology

None performed.

Clinical Signs

Abnormalities of the skin at the application site were noted in female mice in the 12.5 and 25 mg/kg dose groups and in male mice in the 6.25 mg/kg dose group and higher. These abnormalities included irritation or redness, thickening, scaliness, and/or bleaching.

Necropsy Data

The only lesion occurring in more than 1 of 10 mice was depigmentation of hair at the application site in 25 mg/kg males and females (6 and 4 of 10 respectively). It should be noted that in the report from the original laboratory, scars, crusts, and depigmentation of hair are interpreted as evidence of skin inflammation and these were attributed to Benzethonium Chloride (p. 30 and 33). However, the table of gross lesions (p. 33) indicates a scar in only 1 female mouse at 1.56 mg/kg and in no others. It would seem

more appropriate to consider this an incidental lesion inasmuch as it occurred in none of the other 99 treated animals at up to 16 times that concentration.

Histopathology

The OP reported significant lesions only in the skin. These were related to application of the test chemical, occurring in increasing incidence and severity with increasing concentrations. The QAP and the PWG are in essential agreement with this. As for the rats, the OP apparently did not diagnose the proliferative lesions (acanthosis, hyperkeratosis) when necrotizing inflammation was diagnosed. This was not specified in the narrative. It resulted in misleading tabular results showing decreased hyperkeratosis and acanthosis in the 25 mg/kg male and female groups. The QAP rightfully pointed this out and diagnosed the component parts of the lesions in great detail (up to 9 diagnoses for the same section). The PWG Chairperson's initial review combined the proliferative lesions under hyperplasia, epithelial, and the inflammation either as inflammation necrotizing, chronic or chronic, active, or as inflammation, chronic, or chronic, active. In retrospect, the chronic and chronic, active modifiers could be combined under chronic. In general, all lesions occurred with less severity than in rats and, in particular, necrosis and/or ulceration were of lower incidence and severity.

As for rats, there was no description of the microscopic lesions.

Although hypercellularity of the bone marrow in 25 mg/kg rats was noted it was not detected in the mice.

Summary

The PWG considered the 3.12 dose level to be appropriate for the chronic study. This was based primarily on the occurrence of necrosis in the epidermis in 6.25 and 12.5 mg/kg male groups. This occurred, however, in only 1 mouse in each of these groups and was of minimal severity. The possibility of a higher dose might be considered although the male group at 6.25 mg/kg had a 14% decrease in weight gain.

The PWG considered the pathology narrative to be unsatisfactory owing to lack of lesion description or clarification of the decrease of hyperkeratosis and acanthosis in the 25 mg/kg groups.

Action Items

1. The PWG Chairperson will review again the lesions in the treated skin, classify them under chronic inflammation, necrosis, ulcer, and epithelial hyperplasia, and produce tables useful for dose-setting for the chronic.

2. The slides will be returned to the OP for reconsideration of the sections of treated skin.

Action Taken

The following summary tables and individual tables from re-evaluation of treated skin by the PWG Chairperson are provided. It should be noted that the full PWG rated the severity of the lesions somewhat lower than the PWG Chairperson (0 to 1 degree lower)

SUMMARY TABLE

Male Mice

mg/kg	<u>0.0</u>	<u>1.56</u>	<u>3.12</u>	<u>6.25</u>	<u>12.5</u>	<u>25.0</u>
Inflammation, Chronic Dermis	1/10*	2/10	3/10	6/10	9/10	10/10
	0.0**	1.0	1.0	1.0	1.2	2.0
Necrosis	0/10	0/10	0/10	1/10	1/10	5/10
	0.0	0.0	0.0	1.0	1.0	1.6
Ulcer	0/10	0/10	0/10	0/10	0/10	1/10
	0.0	0.0	0.0	0.0	0.0	1.0
Hyperplasia, Epithelial	0/10	9/10	8/10	9/10	9/10	10/10
	0.0	1.0	1.0	1.1	1.6	2.2

Female Mice

mg/kg	<u>0.0</u>	<u>1.56</u>	<u>3.12</u>	<u>6.25</u>	<u>12.5</u>	<u>25.0</u>
Inflammation, Chronic Dermis	1/10*	6/10	8/10	8/10	10/10	10/10
	1.0**	1.0	1.0	1.1	1.6	1.9
Necrosis	0/10	0/10	0/10	0/10	0/10	2/10
	0.0	0.0	0.0	0.0	0.0	1.0
Ulcer	0/10	0/10	0/10	0/10	0/10	0/10
	0.0	0.0	0.0	0.0	0.0	0.0
Hyperplasia, Epithelial	0/10	9/10	10/10	10/10	10/10	10/10
	0.0	1.0	1.0	1.0	1.5	2.0

* No. affected/No. in group

** Average degree of severity

0-Within Normal Limits

1-Minimal

2-Mild

3-Moderate

4-Marked

INDIVIDUAL ANIMAL TABLE

MALE MICE

Control										
Animal #	1	2	3	4	5	6	7	8	9	10
Inflammation, Chronic, Dermis	0	0	0	0	0	0	0	0	0	0
Necrosis	0	0	0	0	0	0	0	0	0	0
Ulcer	0	0	0	0	0	0	0	0	0	0
Hyperplasia, Epithelial	0	0	0	0	0	0	0	0	0	0
1.56 mg/kg										
Animal #	11	12	13	14	15	16	17	18	19	20
Inflammation, Chronic, Dermis	0	0	1	0	0	1	0	0	0	0
Necrosis	0	0	0	0	0	0	0	0	0	0
Ulcer	0	0	0	0	0	0	0	0	0	0
Hyperplasia, Epithelial	1	0	1	1	1	1	1	1	1	1
3.12 mg/kg										
Animal #	21	22	23	24	25	26	27	28	29	30
Inflammation, Chronic, Dermis	0	1	1	1	0	0	0	0	0	0
Necrosis	0	0	0	0	0	0	0	0	0	0
Ulcer	0	0	0	0	0	0	0	0	0	0
Hyperplasia, Epithelial	1	1	1	1	1	0	1	1	0	1

* Average degree of severity
 0-Within Normal Limits
 1-Minimal
 2-Mild
 3-Moderate
 4-Marked

6.25 mg/kg										
Animal #	31	32	33	34	35	36	37	38	39	40
Inflammation, Chronic, Dermis	1	0	1	1	1	0	1	1	0	0
Necrosis	0	0	0	1	0	0	0	0	0	0
Ulcer	0	0	0	0	0	0	0	0	0	0
Hyperplasia, Epithelial	1	1	1	1	2	0	1	1	1	1

12.5 mg/kg										
Animal #	41	42	43	44	45	46	47	48	49	50
Inflammation, Chronic, Dermis	1	2	1	1	0	1	1	1	1	2
Necrosis	0	0	0	0	0	0	0	1	0	0
Ulcer	0	0	0	0	0	0	0	0	0	0
Hyperplasia, Epithelial	2	2	1	1	0	1	1	2	2	2

25 mg/kg										
Animal #	51	52	53	54	55	56	57	58	59	60
Inflammation, Chronic, Dermis	2	2	2	2	2	2	2	3	1	2
Necrosis	0	0	2	2	0	2	0	1	0	1
Ulcer	0	0	0	0	1	0	0	0	0	0
Hyperplasia, Epithelial	2	3	2	3	2	2	2	2	2	2

* Average degree of severity
 0-Within Normal Limits
 1-Minimal
 2-Mild
 3-Moderate
 4-Marked

FEMALE MICE

Control

Animal #	61	62	63	64	65	66	67	68	69	70
Inflammation, Chronic, Dermis	0	0	0	0	0	0	0	0	0	1
Necrosis	0	0	0	0	0	0	0	0	0	0
Ulcer	0	0	0	0	0	0	0	0	0	0
Hyperplasia, Epithelial	0	0	0	0	0	0	0	0	0	0

1.56 mg/kg

Animal #	71	72	73	74	75	76	77	78	79	80
Inflammation, Chronic, Dermis	1	1	0	0	0	1	1	1	1	0
Necrosis	0	0	0	0	0	0	0	0	0	0
Ulcer	0	0	0	0	0	0	0	0	0	0
Hyperplasia, Epithelial	1	1	0	1	1	1	1	1	1	1

3.12 mg/kg

Animal #	81	82	83	84	85	86	87	88	89	90
Inflammation, Chronic, Dermis	1	0	1	1	1	1	1	0	1	1
Necrosis	0	0	0	0	0	0	0	0	0	0
Ulcer	0	0	0	0	0	0	0	0	0	0
Hyperplasia, Epithelial	1	1	1	1	1	1	1	1	1	1

* Average degree of severity
0-Within Normal Limits
1-Minimal
2-Mild
3-Moderate
4-Marked

6.25 mg/kg										
Animal #	91	92	93	94	95	96	97	98	99	100
Inflammation, Chronic, Dermis	1	1	0	1	1	1	0	1	1	2
Necrosis	0	0	0	0	0	0	0	0	0	0
Ulcer	0	0	0	0	0	0	0	0	0	0
Hyperplasia, Epithelial	1	1	1	1	1	1	1	1	1	1

12.5 mg/kg										
Animal #	101	102	103	104	105	106	107	108	109	110
Inflammation, Chronic, Dermis	1	2	2	2	1	2	2	2	1	1
Necrosis	0	0	0	0	0	0	0	0	0	0
Ulcer	0	0	0	0	0	0	0	0	0	0
Hyperplasia, Epithelial	1	2	2	1	1	2	2	2	1	1

25 mg/kg										
Animal #	111	112	113	114	115	116	117	118	119	120
Inflammation, Chronic, Dermis	2	2	2	1	2	1	2	2	2	3
Necrosis	0	0	0	0	0	0	0	0	1	1
Ulcer	0	0	0	0	0	0	0	0	0	0
Hyperplasia, Epithelial	3	2	3	1	2	1	2	2	2	2

* Average degree of severity
 0-Within Normal Limits
 1-Minimal
 2-Mild
 3-Moderate
 4-Marked



DEPARTMENT OF HEALTH & HUMAN SERVICES

Public Health Service

May 2, 1994

National Institutes of Health
National Institute of
Environmental Health Sciences
P.O. Box 12233
Research Triangle Park, N.C. 27709

Mr. Elliot Harrison
Delta Analytical Corporation
7910 Woodmont Ave., Suite 1000
Bethesda, MD 20814

Dear Mr. Harrison,

Enclosed are the NTP/NIEHS mutagenicity data on Benzethonium chloride (CAS # 121-54-0). We have tested this chemical as a coded sample in the Salmonella and in vitro cytogenetics assays. The names of the test laboratories and results appear on the data forms. I have enclosed code sheets and summary protocols for each test system.

The in vitro cytogenetics data have not been published. I request that you not publish these data or include them in any formal report. You may reference the conclusion for these tests as "NTP unpublished results." The references for the published data are as follows:

Zeiger, E., Anderson, B., Haworth, S., Lawlor, T., Mortelmans, K., and Speck, W. (1987) Salmonella mutagenicity tests. III. Results from the testing of 225 chemicals. Environ. Mutagen. 9(Suppl 9): 1-109.

I hope that this information is of use to you.

Sincerely,

A handwritten signature in black ink, appearing to read "Errol Zeiger", is written over a horizontal line.

Errol Zeiger, Ph.D., J.D.
Head, Chemical Selection and
Information Management Office
Environmental Toxicology Program

SALMONELLA PROTOCOL

All chemicals are tested and evaluated as unknowns for their ability to induce gene mutations in Salmonella typhimurium. A detailed protocol of the test system is presented in Haworth et al., Environ. Mutagen. 5(Suppl. 1): 3-142, 1983. Chemicals tested most recently have been tested in this system with minor modifications as indicated below. A preincubation modification of the Salmonella test is used; the test chemical is incubated with the tester strain either in buffer or S9 plus cofactor mix, for 20 minutes at 37°C prior to the addition of soft agar and plating on minimal agar plates. All chemicals are tested both in the absence of metabolic activation and with exogenous metabolic activation (S9) from Aroclor 1254-induced Sprague-Dawley rats and Syrian hamsters, in Salmonella strains TA98, TA100, TA1535, TA1537 and/or TA97. Testing is done either using a series of 4 strains or using a hierarchy of strains. When tested in series all negatives are repeated; all positives are repeated for conditions that elicited the positive response. When tested as a hierarchy, chemicals are tested initially in TA100 and TA98 and repeated if positive. If negative, the chemical is then tested in 2 or 3 of the other strains. If still negative, all strains are retested with a change in the S9 concentration. Each test consists of triplicate plating of concurrent positive and solvent controls and of at least 5 doses of test chemical; the high dose is limited by toxicity or solubility, but not exceeding 10 mg/plate. A positive response is defined as a reproducible, dose related increase in histidine-independent (revertant) colonies. An equivocal response (?) is either a non-dose-related increase or a response that is not reproducible. A chemical is judged positive if a reproducible positive response is observed in any strain/activation combination.

CELLULAR AND GENETIC TOXICOLOGY BRANCH, NTP
SALMONELLA TESTING RESULTS

CAS #: 121-54-0 Benzethonium chloride
ALIQOT: 397650 LAB: CASE WESTERN RESERVE UNIVERSITY
MUTAGENICITY CONCLUSION: -

TA100		SOLVENT: H2O				PROTOCOL: PREINCUBATION							
DOSE		1 NA (-)		2 NA (-)		1 10% HLI (-)		2 10% HLI (-)		1 10% RLI (-)		2 10% RLI (-)	
ug/PLATE		MEAN	SE	MEAN	SE	MEAN	SE	MEAN	SE	MEAN	SE	MEAN	SE
0.000		90	1.8	101	9.0	109	7.1	152	5.2	128	9.3	142	4.7
0.010		87	0.3	92	3.8								
0.030		85	4.2	107	0.3								
0.100		82	5.2	103	5.3								
0.300		94	4.7	99	4.3								
1.000		92	5.0	66	1.7	105	1.5	130	5.9	109	7.4	122	8.0
3.300						100	3.2	133	16.3	121	6.1	132	10.1
10.000						94	2.3	140	14.2	118	13.0	144	6.9
33.000						84	4.1	144	8.8	123	8.7	141	1.3
100.000						34	3.8	143	15.2	128	6.0	t	
POS		936	11.1	1024	18.1	1636	125.5	1021	63.2	1264	205.4	2105	62.1

TA1535		SOLVENT: H2O				PROTOCOL: PREINCUBATION							
DOSE		1 NA (-)		2 NA (-)		1 10% HLI (-)		2 10% HLI (-)		1 10% RLI (-)		2 10% RLI (-)	
ug/PLATE		MEAN	SE	MEAN	SE	MEAN	SE	MEAN	SE	MEAN	SE	MEAN	SE
0.000		18	1.2	4	1.5	17	1.5	6	0.0	25	1.5	7	1.7
0.010		14	1.9	4	0.7								
0.030		16	3.7	4	1.2								
0.100		14	3.2	5	0.7								
0.300		8	1.5	2	1.2								
1.000		8	0.6	2	0.6	18	3.3	4	1.8	16	0.9	5	1.9
3.300						26	1.3	5	1.5	20	2.0	6	2.5
10.000						15	4.2	3	0.9	17	1.5	5	1.7
33.000						9	0.9	4	0.7	8	1.5	4	0.7
100.000						t		t		t		t	
POS		942	36.6	446	18.0	125	10.7	97	15.5	184	15.0	60	8.4

TA1537		SOLVENT: H2O				PROTOCOL: PREINCUBATION							
DOSE		1 NA (-)		2 NA (-)		1 10% HLI (-)		2 10% HLI (-)		1 10% RLI (-)		2 10% RLI (-)	
ug/PLATE		MEAN	SE	MEAN	SE	MEAN	SE	MEAN	SE	MEAN	SE	MEAN	SE
0.000		12	1.5	10	1.2	24	2.4	15	1.5	19	0.3	12	1.5
0.010		9	0.6	13	0.7								
0.030		10	2.8	9	1.5								
0.100		14	1.0	7	0.9								
0.300		16	0.7	7	2.3								
1.000		11	3.8	7	1.2								
3.300						22	2.4	13	1.0	23	3.0	12	1.5
10.000						26	4.3	10	2.1	20	3.4	15	1.7
33.000						14	0.6	9	2.4	13	2.0	7	2.1
100.000						13	1.2	15	2.6	13	2.5	12	1.7
						1	0.7	13	2.5	t		t	
POS		207	94.2	177	104.5	356	21.3	197	15.3	432	29.1	122	43.3

TA98		SOLVENT: H2O				PROTOCOL: PREINCUBATION							
DOSE		1 NA (-)		2 NA (-)		1 10% HLI (-)		2 10% HLI (-)		1 10% RLI (-)		2 10% RLI (-)	
ug/PLATE		MEAN	SE	MEAN	SE	MEAN	SE	MEAN	SE	MEAN	SE	MEAN	SE
0.000		20	1.8	14	4.1	19	2.5	28	3.2	22	3.0	21	0.9
0.010		21	0.7	7	1.2								
0.030		18	1.8	6	0.7								
0.100		19	2.1	7	0.3								
0.300		18	0.9	10	1.8								
1.000		22	1.5	10	4.2	15	1.9	28	4.1	21	2.0	17	1.9
3.300						16	2.9	20	0.7	24	3.2	20	2.4
10.000						16	1.7	20	1.3	24	1.5	16	1.8
33.000						10	2.1	19	2.9	16	0.7	24	1.2
100.000						7	1.2	21	0.9	18	0.7	18	3.5
POS		180	63.8	123	9.0	1152	40.1	719	45.8	872	70.4	1150	21.3

END OF ALIQUOT

IN VITRO CYTOGENETICS PROTOCOL SUMMARY

Chemicals are tested as unknowns for their ability to induce sister chromatid exchanges (SCEs) and chromosome aberrations in Chinese hamster ovary cells. A detailed protocol is presented in Galloway et al., Environ Mutagen. 7:1-51, (1985). Chemicals are tested for both endpoints with and without metabolic activation. The metabolic activation (S9) is derived from the livers of Aroclor 1254-induced male Sprague-Dawley rats. Each test consists of concurrent solvent and positive controls and of at least 3 doses of test chemical; the high dose is limited by toxicity or solubility, but does not exceed 5 mg/ml. The data are statistically analyzed for both trend and peak response. A positive response requires that 2 doses produce a significant effect (SCEs: 20% over control; aberrations: $P < 0.01$); if only one dose or only the trend test is significant, the response is considered weakly positive or equivocal, respectively.

In the SCE assay without S9, the cells are treated with the chemical for 26 hours in McCoy's 5A medium; two hours after the chemical treatment begins bromodeoxyuridine (BU) is added to the culture. After 26 hours the treated medium is removed and replaced with medium containing BU and Colcemid and incubated for 2 hours. Cells are then harvested by mitotic shake-off, fixed, and stained with Hoechst 33258 and Giemsa. If insufficient cells are obtained for scoring, later harvest times are used. In the assay with S9, the cells are incubated with the chemical, serum-free medium, and S9. After 2 hours, the treatment is removed and replaced with BU containing medium for 26 hours. Colcemid is present for the final 2 hours. The same procedure is followed for harvesting and staining as for treatment without S9.

In the chromosome aberration assay without S9, the test chemical is incubated with the cells in McCoy's 5A medium for 8 hours followed by a 2 hour incubation with colcemid. The cells are then harvested, fixed and stained with Giemsa. For the assay with S9, the cells are treated with chemical and S9 for 2 hours. The treatment mixture is removed and the cells are then incubated for 10 hours, with colcemid present for the last 2 hours. The cells are harvested in the same manner as for the treatment without S9. If significant cell cycle delay is seen in either procedure, the cells can be incubated longer prior to addition of colcemid.

CAS #: 121-54-0 CHEMICAL NAME: Benzethonium chloride
ALIQOUT #: 173525 LABORATORY: Columbia University

PUBLICATION:

SISTER CHROMATID EXCHANGES (SCE)
CONCLUSION: -

TRIAL #: 1 ACTIVATION: NA DATE: 08/31/81 PROGRAM CALL: - STAT CALL: - LAB CALL:

	DOSE UG/ML	TOTAL CELLS	NO. OF CHROMO	NO. OF SCES	SCE / CHROMO	SCE / CELL	HRS IN BRDU	% INCR OVER SOL
SOL: H2O		50	1047	434	0.41	8.68	26.0	
TEST	0.9600	50	1049	458	0.44	9.16	26.0	5.33
CONC:	3.0000	50	1050	457	0.44	9.14	26.0	5.00
	9.6000	50	1048	473	0.45	9.46	26.0	8.88
POS: MMC	0.0050	25	523	639	1.22	25.56	26.0	194.76

TREND: 1.198638
PROBABILITY: 0.115334

REMARKS: CONTROL ALSO FOR 113918.

TRIAL #: 1 ACTIVATION: RLI DATE: 08/31/83 PROGRAM CALL: - STAT CALL: - LAB CALL:

	DOSE UG/ML	TOTAL CELLS	NO. OF CHROMO	NO. OF SCES	SCE / CHROMO	SCE / CELL	HRS IN BRDU	% INCR OVER SOL
SOL: H2O		50	1043	369	0.35	7.38	26.0	
TEST	3.0000	50	1048	347	0.33	6.94	26.0	-6.41
CONC:	9.6000	50	1050	359	0.34	7.18	26.0	-3.36
	30.0000	50	1047	367	0.35	7.34	26.0	-0.92
POS: CPA	1.0000	50	1045	790	0.76	15.80	26.0	113.69

TREND: 0.013273
PROBABILITY: 0.494705

REMARKS: CONTROL ALSO FOR 113918.

Study Title

DEVELOPMENTAL TOXICITY STUDY OF HYAMINE® 1622 IN RATS

Data Requirement

U.S. Environmental Protection Agency
Pesticide Assessment Guidelines
Subdivision F, 83-3

Author

John A. Foss, Ph.D.
(Study Director)

Study Completed On

October 26, 1995
(Final Report)

Performing Laboratory

Argus Research Laboratories, Inc.
905 Sheehy Drive
Horsham, Pennsylvania 19044

Sponsor

Lonza Inc.
17-17 Route 208
Fair Lawn, New Jersey 07410


Laboratory Project ID

Argus Research Laboratories, Inc., Protocol Number: 720-002

Statement of No Data Confidentiality Claims

No claim of confidentiality is made for any information contained in this study on the basis of its falling within the scope of FIFRA Section 10(d)(1)(A), (B), or (C).

Submitter/Sponsor:



Joseph R. Robinson 10/24/95
Lonza Inc. Date
Director of Customer Services
Technical, Regulatory, and Customer

GOOD LABORATORY PRACTICE STATEMENT

This study was conducted according to U.S. Environmental Protection Agency (EPA FIFRA) "Good Laboratory Practice Standards; Final Rule" (40 CFR Part 160). Minor deviations from the study protocol and/or Standard Operating Procedures of the Testing Facility are documented in the study record. No deviations existed that affected the validity of the study.

Study Director:

John A. Foss 26 Oct 95
 John A. Foss, Ph.D. Date
 Argus Research Laboratories, Inc.

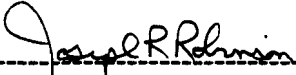
Submitter/Sponsor:

Joseph R. Robinson 10/24/95
 Joseph R. Robinson Date
 Lonza Inc.
 Director of Customer Services
 Technical, Regulatory, and Customer

Flagging Statement

I have applied the criteria of 40 CFR 158.34 for flagging studies for potential adverse effects to the results of the attached study. This study neither meets nor exceeds any of the applicable criteria.

Submitter/Sponsor:

 10/24/95

Joseph R. Robinson Date
Lonza Inc.
Director of Customer Services
Technical, Regulatory, and Customer



Argus Research Laboratories, Inc.
 905 Sheehy Drive, Building A
 Horsham, Pennsylvania 19044
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PROTOCOL 720-002: DEVELOPMENTAL TOXICITY STUDY OF
 HYAMINE® 1622 IN RATS

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TITLE: DEVELOPMENTAL TOXICITY STUDY OF HYAMINE® 1622 IN RATS

ARGUS RESEARCH LABORATORIES, INC.,
PROTOCOL NUMBER: 720-002

I. SUMMARY

Mated Crl:CD®BR VAF/Plus® (Sprague-Dawley) female rats (25 animals per dosage group) were administered 0(Vehicle), 10, 30, 100 and 170 mg/kg/day dosages of Hyamine® 1622 on days 6 through 15 of presumed gestation. The vehicle was reverse osmosis processed deionized water. The test substance and vehicle were administered perorally by gavage at a dosage volume of 10 mL/kg with the actual volume based on the most recent body weight of each rat.

The female rats and male breeder rats were evaluated for health status during the acclimation period. During the presumed gestation period, the female rats were observed for viability and overt signs of toxicity at least twice each day. The rats were also given a detailed physical examination that included examinations for clinical signs of toxicity, abortions, premature deliveries and deaths before and approximately one hour after administration of the test substance and vehicle on days 6 through 15 of presumed gestation. During the postdosage period (days 16 through 20 of presumed gestation) these detailed physical examinations were conducted once daily. Body weights and feed consumption values were recorded on days 0, 6, 9, 12, 15, 16, 18 and 20 of presumed gestation.

Rats that were found dead or rats that prematurely delivered a litter were sacrificed and necropsied on the day the event occurred. All surviving rats were sacrificed by carbon dioxide asphyxiation on day 20 of presumed gestation, and a gross necropsy of the thoracic, abdominal and pelvic viscera was performed. The number of corpora lutea in each ovary was recorded. The uterus of each rat was excised, weighed and examined for pregnancy,

number and distribution of implantations, live and dead fetuses and early and late resorptions. All fetuses were subsequently weighed and examined for sex and gross external alterations. Approximately one-half of the fetuses in each litter were examined for soft tissue alterations using the microdissection technique of Staples with minor modifications. In addition, the heads of these fetuses were fixed in Bouin's solution and subsequently examined by free-hand sectioning. The other fetuses in each litter were examined for skeletal alterations after staining with alizarin red S.

Four rats in the high dosage group (170 mg/kg/day) were found dead on gestational days (GD) 11, 12, 13 or 16. These deaths were considered related to the administration of the test substance. One dam in the low dosage group (10 mg/kg/day) was sacrificed because it prematurely delivered on GD 20; this death was a non-dosage-dependent event unrelated to the test substance. All other dams survived to scheduled sacrifice.

An increased number of rats in the 170 mg/kg/day dosage group had abnormal feces (soft feces, liquid feces, or no feces), alopecia on the underside, excess salivation, urine-stained abdominal fur and fecal-stained fur. Other observations, such as a red substance around the nose or on both paws and forelimbs, being cold to touch, ptosis, impaired righting reflex, pale appearance, brown perivaginal substance, red substance in cage pan, and a red substance on the abdominal fur occurred only in the rats that died. Other treatment-related changes for dams in the high dosage group included dyspnea and rates, no other biologically significant treatment-related clinical observations were noted for dams in this study.

Dams in the 170 mg/kg/day dosage group lost body weight from GD 6 and 9, and body weight gains on GD 9 to 12, for the entire dosage period (calculated as GD 6 to 16), and for the cumulative intervals of GD 6 to 20 and GD 0 to 20 were also reduced. Average absolute body weights in this group were reduced on GD 9 and thereafter. Gravid uterine weights were comparable among the dosage groups indicating that the body weight reductions were not associated with reductions in gravid uterine weight.

Maternal feed consumption values (g/day) were reduced for dams in the 170 mg/kg/day dosage group throughout the dosage period and for the cumulative intervals (calculated as GD 6 to 20 and GD 0 to 20). No other treatment-related changes in maternal feed consumption values were observed.

Maternal necropsy findings were for the most part limited to those rats that died during the study and generally involved changes in the gastrointestinal tract. Those included gas or fluid-filled, thin walls, no mucosal folds, and/or black spots in the stomach, intestines or cecum.

There were no treatment-related differences in the litter averages for corpora lutea, implantations, litter size, live and dead fetuses, early and late resorptions, percent dead or resorbed conceptuses per litter, percent male fetuses per litter and live fetal body weights. A small number of fetal alterations (malformations and variations) were observed in each group, including the vehicle control. None of these alterations was considered related to the test substance. Analyses of the average numbers of fetal ossification sites per litter did not reveal biologically important or statistically significant differences among the five dosage groups.

Hyamine® 1622 was not a developmental toxicant in this study. Based upon the data collected in this study, the maternal no-observable-adverse-effect-level (NOAEL) and the developmental NOAEL for the test substance are 100 mg/kg/day and greater than 170 mg/kg/day, respectively.

Alan M. Hoberman 26 Oct 95

Alan M. Hoberman, Ph.D., DABT Date
Director of Research

Mildred S. Christian 26 Oct 95

Mildred S. Christian, Ph.D., ATS Date
Executive Director of Research

John A. Foss 26 Oct 95

John A. Foss, Ph.D. Date
Group Leader and Study Director

II. GENERAL INFORMATION

Sponsor:

Lonza Inc., 17-17 Route 208, Fair Lawn, New Jersey 07410

Testing Facility:

Argus Research Laboratories, Inc., 905 Sheehy Drive, Horsham,
Pennsylvania 19044-1297

Study Number:

720-002

Purpose of the Study:

The purpose of this study was to evaluate the potential for Hyamine® 1622 to produce developmental toxicity (embryo-fetal toxicity and teratogenesis) when administered orally via gavage to Crl:CD®BR VAF/Plus® presumed pregnant rats.

Regulatory Compliance:

The standards of the U.S. Environmental Protection Agency (EPA)^(1,2) were used as the basis for study design and compliance with Good Laboratory Practices (GLPs). There were no significant deviations from the GLP Regulations that affected the quality or integrity of the study. Quality Assurance Unit findings derived from the inspections during the conduct of this study are documented and have been provided to the Study Director and the Testing Facility Management.

Study Director:

John A. Foss, Ph.D. (Group Leader)

Technical Performance:

John F. Barnett, B.S. (Director of Laboratory Operations)
Eileen M. Tlush, B.S. (Supervisor)
Barbara J. Roe, B.S. (Laboratory Technician)

Report Preparation:

John A. Foss, Ph.D. (Group Leader)
 Anthony S. Conlon, B.S. (Supervisor)
 Cheryl L. Van, B.A. (Senior Methods Coordinator)
 Georgia Y. Burnett, A.A.S. (Administrative Assistant)

Report Review:

Alan M. Hoberman, Ph.D., DABT (Director of Research)
 Mildred S. Christian, Ph.D., ATS (Executive Director of Research).

Date Protocol Signed:

May 18, 1995

Dates of Technical Performance:

Arrival Date	08 MAY 95
Acclimation Period	08 MAY 95 - 22 MAY 95
Cohabitation Period	22 MAY 95 PM - 27 MAY 95 AM
Day 0 of Presumed Gestation	23 MAY 95 - 26 MAY 95
Dosage Period (Days 6 through 15 of presumed gestation)	29 MAY 95 - 10 JUN 95
Caesarean-Sectioning Period (Day 20 of presumed gestation)	12 JUN 95 - 15 JUN 95

Records Maintained:

The original report, raw data and reserve samples of the test substance and vehicle are retained in the archives of Argus Research Laboratories, Inc. Any specimens are retained in the archives of the Testing Facility for one year after mailing of the draft final report, after which time the Sponsor will be contacted to determine the disposition of these materials. Unused test substance was retained at the Testing Facility for possible additional studies.

III. DESCRIPTION OF TEST PROCEDURES

A. Test Substance Information:

A.1. Description:

Chemical Name: Benzethonium Chloride

Synonym: Hyamine® 1622

Source: Lonza Inc., 79 Route 22 East, Annandale, New Jersey 08801

CAS Registry Number: 121-54-0

Sponsor ID: Hyamine® 1622 (Lonza TRCS Number: 40109)

Argus Research Laboratories, Inc. ID: Hyamine® 1622

Percent Active Ingredient: 99.3^a

Description: Homogenous, fine, white powder free from visible foreign matter

Amount Received: 5 bottles (1.00 kg net each)

A.2. Lot Number:

40109

A.3. Date Received and Storage Conditions:

The test substance was received on March 8, 1995, and stored at room temperature in an environmentally controlled area.

A.4. Special Handling Instructions:

Standard safety precautions (use of protective clothing, gloves, dust-mist respirator, safety goggles or safety glasses and a face-shield) were taken when handling the test substance.

B. Vehicle Information:

B.1. Description:

Reverse osmosis processed deionized water (R.O. deionized water)

-
- a. Analyses conducted before receipt at the Testing Facility. On October 10, 1995, a 10 g sample of the bulk test substance was sent at ambient temperature to the Sponsor to confirm the purity of the test substance after storage at the Testing Facility.

B.2. Analysis of Purity:

Neither the Sponsor nor the Study Director was aware of any potential contaminants likely to be present in the vehicle that would interfere with the results of this study.

C. Preparation of Dosing Solutions:

Each dosing solution was prepared by combining the appropriate amounts of the test substance (grams) and R.O. deionized water to obtain the required volume of solution.

The amount of the test substance used in the preparation of the dosing solutions was not adjusted for the percent active ingredient since the test substance is >99% pure. Detailed preparation procedures are attached to the protocol (see APPENDIX 1). Storage stability analyses conducted prior to the study showed that the dosing solutions were stable for at least 21 days when stored at room temperature. Consequently, dosing solutions were only prepared once for the study (see APPENDIX 3).

C.1. Reserve Sample Information:

Reserve samples (10 grams) of the test substance and vehicle were retained before dosage began on May 23, 1995. These samples are stored at ambient temperature in the Testing Facility archives.

D. Analyses of Dosing Solutions:**D.1. Homogeneity, Stability and Concentration Analyses:**

Before initiation of this developmental toxicity study, trial batches were prepared at concentrations of 0.3, 15 and 20 mg/mL in order to assess homogeneity and stability of the dosing solutions across the range of concentrations to be used in the study. Samples (6 mL each) were taken on the day of preparation from the top, middle and bottom of the mixing vessel for each concentration to evaluate homogeneity. The solutions were then stored at room temperature and additional samples (6 mL; one each from the top, middle and bottom of the vessel for each concentration) were taken at 14 days and again at 21 days after preparation to evaluate the stability and longer term homogeneity of the dosing solutions. Each sample was divided into three approximately equal parts; two parts were refrigerated (2°C to 8°C) and shipped to Lancaster Laboratories, Inc. (Lancaster, Pennsylvania) for analysis, and one part was retained refrigerated at the Testing Facility as a backup. The results of the analyses of these concentrations indicated that the formulations were homogeneous and stable at room temperature for at least

21 days, but only the results for the 0.3 mg/mL and 20 mg/mL concentrations are tabulated in the analytical report (see APPENDIX 3) because those concentrations bracketed the range of concentrations given to the rats in this study.

Dosing solutions prepared for administration to the rats were analyzed for the concentration of test substance before being administered to the rats. One sample (6 mL) was taken from the middle of each concentration on the day of preparation. Each sample was divided into three approximately equal parts (2 mL each); two parts were refrigerated and shipped to Lancaster Laboratories, Inc., for analysis, and one part was retained refrigerated at the Testing Facility as a backup. The results of these analyses indicated that the formulations used for dosage contained the appropriate concentrations of the test substance (see APPENDIX 3).

E. Test System:

E.1. Species:

Rat

E.2. Strain:

CrI:CD®BR VAF/Plus®

E.3. Supplier (Source):

Charles River Laboratories, Inc., Portage, Michigan

E.4. Sex:

Female (Note: Male rats were used only for the purposes of breeding and are not considered part of the Test System.)

E.5. Rationale for Test System:

The CrI:CD®BR VAF/Plus® (Sprague-Dawley) rat was selected as the Test System because: 1) it is one mammalian species accepted and widely used throughout industry for nonclinical studies of developmental toxicity (embryo-fetal toxicity/teratogenicity); 2) this strain has been demonstrated to be sensitive to developmental toxins; and 3) historical data and experience exist at the Testing Facility⁽³⁻⁵⁾.

E.6. Test System Data:

Number of Rats	165
Approximate Date of Birth	05 MAR 95
Approximate Age at Arrival	65 days
Weight (g) on the Day After Arrival	183 - 227
Weight (g) at Study Assignment	217 - 259

E.7. Breeder Male Rat Data:

Number of Rats	170
Approximate Date of Birth	14 JAN 95
Approximate Age at Arrival	74 days
Weight (g) on the Day After Arrival	283 - 348
Weight (g) at Cohabitation	443 - 618

E.8. Pre-Study Health Screen:

After arrival, five female rats were randomly selected and examined by the Testing Facility Staff Veterinarian for general health. Fecal samples were collected from each of the five rats and examined for parasites. The selected rats were sacrificed by carbon dioxide asphyxiation and a blood sample (4.0 to 5.0 mL) was collected from the vena cava. The blood was centrifuged and the resulting serum was diluted 1:4 with phosphate buffered saline. The serum dilution was shipped (on dry ice) to Charles River Laboratories, Inc., (Diagnostic Lab, Wilmington, Massachusetts) for routine serology testing. A gross necropsy of the thoracic, abdominal and pelvic viscera was performed.

Testing Facility male breeder rats were also examined by a veterinarian before mating. Three male rats from the breeding colony were selected for fecal parasite evaluations. The results of the pre-study health screen indicated that the animals were free from infectious disease and physical abnormality.

F. Husbandry:**F.1. Research Facility Registration:**

USDA Registration No. 23-R-099 under the Animal Welfare Act, 7 U.S.C. 2131 *et seq.*

F.2. Study Room:

The study room was maintained under conditions of positive airflow relative to a hallway and independently supplied with a minimum of ten changes per hour of 100% fresh air that had been passed through 99.97% HEPA filters (Airo

Clean® room). Room temperature and humidity were recorded constantly throughout the study. Room temperature was targeted at 70°F to 78°F ($\pm 2\%$); relative humidity was targeted at 40% to 70% ($\pm 3\%$).

F.3. Housing:

Rats were individually housed except during the cohabitation period. During cohabitation, each pair of male and female rats was housed in the male rat's cage. All cage sizes and housing conditions were in compliance with the *Guide for the Care and Use of Laboratory Animals*, NIH Publication No. 86-23⁽⁶⁾.

F.4. Lighting:

An automatically-controlled fluorescent light cycle was maintained at 12-hours light:12-hours dark, with each dark period beginning at 1900 hours EST.

F.5. Sanitization:

Cage pan liners were changed approximately three times each week. Cages were changed approximately every other week.

F.6. Feed:

Rats were given *ad libitum* access to Certified Rodent Diet® #5002 (meal, Purina Mills, Inc.) in individual feeders.

F.7. Feed Analysis:

Analyses were routinely performed by the feed supplier. No contaminants in the feed or deviations from expected nutritional requirements were detected by these analyses. Copies of the results of the feed analyses are available in the raw data and in APPENDIX 4.

No agent present in the feed was considered to interfere with the results of this study.

F.8. Water:

Local water that had been processed by passage through a reverse osmosis membrane (R.O. water) was available to the rats *ad libitum* from an automatic watering system and/or individual water bottles. Chlorine was added to the processed water as a bacteriostat.

F.9. Water Analysis:

The processed water is analyzed twice annually for possible chemical contamination (Lancaster Laboratories, Inc., Lancaster, Pennsylvania) and monthly for possible bacterial contamination (Analytical Laboratories, Inc., Chalfont, Pennsylvania) (see APPENDIX 2, item 1). Copies of the results of the water analyses are available in the raw data and in APPENDIX 5.

No agent present in the water was considered to interfere with the results of this study.

G. Animal Identification and Assignment to Groups:**G.1. System of Identification:**

Cage tags were marked with the study number, permanent rat number, sex, test substance identification and dosage level. Each rat was individually identified with a Monel® self-piercing ear tag (Gey Band and Tag Co., Inc., No. MSPT 20101) inscribed with the rat's designated unique permanent number.

G.2. Method of Randomization and Assignment to Groups:

Upon arrival all rats were assigned to individual housing based on computer-generated random units. After acclimation, consecutive order was used to assign virgin female rats to cohabitation with breeder male rats (one male rat per female rat). Only rats that appeared healthy and had normal weight gain during the acclimation period were considered suitable for cohabitation. Following cohabitation, female rats were assigned to one of five dosage groups (Groups I through V), twenty-five rats per dosage group, using a computer-generated (weight-ordered) randomization procedure based on body weights recorded on day 0 of presumed gestation.

H. Cohabitation:

After acclimation, 165 healthy virgin female rats were placed into cohabitation with 165 breeder male rats (one male rat per female rat in the male rat's cage). Female rats with spermatozoa observed in a smear of the vaginal contents or a copulatory plug *in situ* were considered to be at day 0 of presumed gestation and returned to individual housing.

I. Methods:

I.1. Experimental Design:

<u>Group</u>	<u>Number of Rats</u>	<u>Dosage (mg/kg/day)^a</u>	<u>Concentration (mg/mL)^a</u>	<u>Volume (mL/kg)</u>	<u>Assigned Number</u>
I	25	0(Vehicle)	0	10	6801 - 6825
II	25	10	1.0	10	6827 - 6850, 3100 ^b
III	25	30	3.0	10	6851 - 6875
IV	25	100	10.0	10	6876 - 6900
V	25	170	17.0	10	6901 - 6925

- a. Dosage calculations were not adjusted for the percent active ingredient since the test substance is >99% pure.
- b. Before dosage administration on day 6 of presumed gestation (May 29, 1995), Group II rat 6826 was excluded from study because of adverse clinical signs and replaced with rat 3100.

I.2. Rationale for Dosage Selection:

Doses were selected based on the results of a short-term oral toxicity study and a dose range-finding teratology study in rats (Argus Research Laboratories, Inc., Protocol 720-002P). In the oral toxicity study, rats received either one dose of the test substance at dose levels 50, 100, 200, 400 and 800 mg/kg or five consecutive daily doses of the test substance of dose levels of 100, 200 and 300 mg/kg/day. The test substance was administered in aqueous solutions that ranged in concentration from 1% to 16% for the single dose phase and 1% to 3% for the repeated dose phase. A single dose of 400 or 800 mg/kg produced clear toxicity as evidenced by clinical signs of toxicity, body weight loss and decreased food consumption. Following repeated dosing, 200 and 300 mg/kg/day resulted in mortality (1/3 animals in each group), clinical signs of toxicity, body weight loss, decreased food consumption and necropsy changes in the GI tract (i.e., fluid or gas filled, distended, thin walled, no mucosal folds). Treatment-related effects for the animals in the 100 mg/kg/day group were limited to clinical signs of toxicity (loose or liquid feces) and a small loss of body weight during the first four days of treatment.

In the dose range-finding developmental toxicity study, the test substance was administered orally (gavage) at doses of 3, 10, 30, 100 and 150 mg/kg/day on gestation days (GD) 6 to 15. These corresponded to dose solution concentrations that range from 0.3% to 1.5 %. Evidence of maternal toxicity was observed only in the 150 mg/kg/day group and was generally restricted to the early part of the dosage period. Effects included clinical signs of toxicity (loose or liquid feces) and small reductions in body weight gain and food consumption. There were no treatment-related changes in litter parameters or

effects observed at Caesarean-section. There were no gross external alterations that were attributed to treatment with the test substance.

Based on the results of these studies, dose levels of 10, 30, 100 and 170 mg/kg/day were selected for the full developmental toxicity study. These correspond to dose solution concentrations of 0.1, 0.3, 1.0 and 1.7%, respectively. A high dose of 170 mg/kg/day was expected to be adequate to satisfy the requirement to see clear evidence of maternal toxicity. The lower dose levels of 10 and 30 mg/kg/day were not expected to produce developmental or maternal effects. The intermediate dose of 100 mg/kg/day was expected to produce either no effect or a minimum degree of toxicity in the dams and no developmental effects.

I.3. Administration of the Test Substance:

I.3.1. Route of Administration:

The test substance was administered orally via gavage as a single daily dose using 2- or 3-inch long 16- or 18-gauge stainless steel gavage needles attached to a 5 cc disposable syringe. The dosage volume in all groups was 10 mL/kg/day with the actual volume based on the most recent body weight of each rat.

I.3.2. Rationale for Route and Method of Administration:

The oral route and gavage method were selected for use because the oral route is one possible route of human exposure and gavage is a suitable method for accurately administering a perioral dose.

I.3.3. Frequency of Administration:

The female rats were given the test substance once daily on days 6 through 15 of presumed gestation, the period of organogenesis. Dosages were given at approximately the same time each day.

I.3.4. Length of Study:

Approximately 4 weeks

I.4. In-Life Observations and Measurements:

The female rats were observed for viability at least twice each day of the study and for general appearance at least weekly during acclimation and on day 0 of presumed gestation. The rats were also examined for clinical observations of effects of the test substance, abortions, premature deliveries and deaths

before and approximately one hour after administration (days 6 through 15 of presumed gestation). These observations were conducted once daily during the postdosage period (days 16 through 20 of presumed gestation).

Body weights were recorded at least once weekly during acclimation. Body weights and feed consumption values were recorded on days 0, 6, 9, 12, 15, 16, 18 and 20 of presumed gestation (see APPENDIX 2, item 2).

1.5. Gross Necropsy:

Rats that were found dead or delivered a litter and were moribund sacrificed were necropsied on the day the event occurred using the procedures described below. Tissues with gross lesions were retained in neutral buffered 10% formalin. Photographs of gross lesions are available in the raw data.

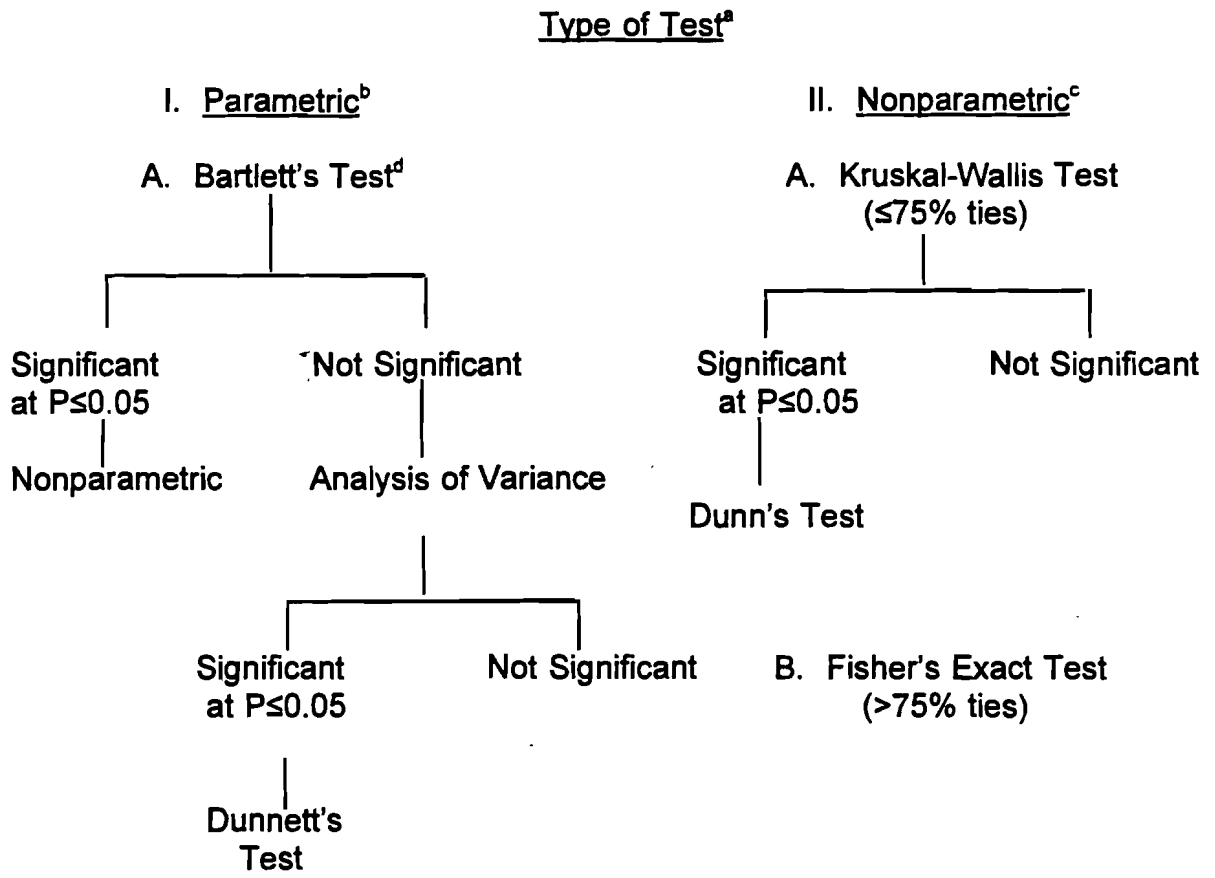
All surviving rats were sacrificed by carbon dioxide asphyxiation on day 20 of presumed gestation, and a gross necropsy of the thoracic, abdominal and pelvic viscera was performed. To confirm the pregnancy status, uteri from rats that appeared nonpregnant were stained with 10% ammonium sulfide to confirm the absence of implantation sites.⁽⁷⁾ Tissues with gross lesions were preserved in neutral buffered 10% formalin for possible future evaluation; all other tissues were discarded (see APPENDIX 2, item 3). Photographs of gross lesions were prepared and are available in the raw data.

The number of corpora lutea in each ovary was recorded. The uterus of each rat was excised, weighed and examined for pregnancy, number and distribution of implantations, live and dead fetuses and early and late resorptions (see APPENDIX 2, item 4).

Each fetus was removed from the uterus and individually identified with a tag noting the study number, litter, uterine distribution and fixative. All fetuses were subsequently weighed and examined for sex and gross external alterations. One dead fetus was identified and was weighed and retained in Bouin's solution for possible future evaluation. Live fetuses were sacrificed according to the Standard Operating Procedures of the Testing Facility. Approximately one-half of the fetuses in each litter were examined for soft tissue alterations using a variation of the microdissection technique of Staples⁽⁸⁾ (see APPENDIX 2, item 5). The heads of these fetuses were fixed in Bouin's solution and subsequently examined by free-hand sectioning; sections were stored in alcohol. The remaining portion of each fetus was eviscerated and stored in alcohol. The remaining fetuses in each litter were examined for skeletal alterations after staining with alizarin red S⁽⁹⁾. These latter fetuses were initially fixed in alcohol; skeletal preparations were retained in glycerine. Representative photographs of fetal gross, soft tissue and skeletal alterations were prepared and are available in the raw data.

I.7. Statistical Analysis:

The following schematic represents the statistical analyses of data:



III. Test for Proportion Data

Variance Test for Homogeneity
of the Binomial Distribution

-
- a. All tests evaluated at P≤0.05 to P≤0.01.
 - b. Used only to analyze data with homogeneous variance.
 - c. Proportion data are not included in this category.
 - d. Test for homogeneity of variance.

Clinical observation and other proportion data were analyzed using the Variance Test for Homogeneity of the Binomial Distribution⁽¹⁰⁾.

Continuous data (e.g., maternal body weights, body weight changes, feed consumption values and litter averages for percent male fetuses, percent dead or resorbed conceptuses, fetal body weights, fetal anomaly data and fetal ossification site data) were analyzed using Bartlett's Test of Homogeneity of Variances⁽¹¹⁾. An Analysis of Variance⁽¹²⁾ was performed if Bartlett's Test was not significant ($P > 0.05$). If the Analysis of Variance was significant ($P \leq 0.05$), Dunnett's Test⁽¹³⁾ was used to identify the statistical significance of the individual groups. If the Analysis of Variance was not appropriate [i.e., Bartlett's Test was significant ($P \leq 0.05$)], the Kruskal-Wallis Test⁽¹⁴⁾ was used, when less than or equal to 75% ties were present. In cases where the Kruskal-Wallis Test was statistically significant ($P \leq 0.05$), Dunn's Method of Multiple Comparisons⁽¹⁵⁾ was used to identify the statistical significance of the individual groups. If there were greater than 75% ties, Fisher's Exact Test⁽¹⁶⁾ was used to analyze the data.

Count data obtained at Cesarean-sectioning of the dams were evaluated using the procedures described above for the Kruskal-Wallis Test⁽¹⁴⁾.

IV. RESULTS

The following text refers to the dosage groups as Groups I through V. These represent the vehicle control group (Group I), the 10 mg/kg/day dosage group (Group II), the 30 mg/kg/day dosage group (Group III), the 100 mg/kg/day dosage group (Group IV) and the 170 mg/kg/day dosage group (Group V).

A. Analytical Chemistry

A detailed description of the methods and results of the analytical chemistry evaluations for the 0.3 and 20 mg/mL formulations are presented in the Analytical Report (APPENDIX 3). The methods and results of the homogeneity and stability evaluations for the 15.0 mg/mL solutions are presented with the report for the Hyamine® 1622 dose range-finding developmental toxicity study (Argus Protocol 720-002P).

Homogeneity and stability analyses performed on batches of solutions that bracketed the low and high concentrations used in the study indicated that the test substance was uniformly distributed and stable for at least 21 days when stored at room temperature in closed containers. The relative standard deviation for the homogeneity analyses of the two concentrations tested (0.3 mg/mL and 20 mg/mL) ranged from 0.4% to 2.6%. The analytical values for the stability tests for both solutions ranged from 98% to 107% of the day 0 values when assayed 14 and 21 days after preparation. Concentration verification analysis for the solutions prepared for the study ranged from 101% to 107% of nominal for the four concentrations containing the test substance. No test substance was detected in the dose solution prepared for the vehicle control group.

B. Mortality

Mortality is summarized in Table 1. Individual animal data for clinical signs of toxicity, body weights, feed consumption values and necropsy observations are summarized in Tables 13 through 16.

Four Group V rats were found dead on gestational days (GD) 11, 12, 13 or 16. These deaths were considered related to the test substance based on the signs of toxicity observed when these rats were alive and the necropsy findings. One Group II dam was sacrificed because it prematurely delivered on GD 20; this death was a non-dosage-dependent event unrelated to the test substance. All other dams survived to scheduled sacrifice. Observations for the dams that died or prematurely delivered are described below.

B.1. Deaths Attributed to the Test Substance

Group V dam 6910 was found dead on GD 11 approximately 3.5 hours after dosage. This rat was cold to touch (GD 10–11) and had excess salivation (GD 8), rales (GD 9–11), impaired righting reflex (GD 11), soft feces (GD 7), liquid feces (GD 8–10), no feces in the cage pan (GD 10–11) and urine-stained abdominal fur, red perinasal substance, and a red substance on the forelimbs and paws (GD 9–11). This dam had the lowest body weight value in the group on day 9 of gestation. Necropsy revealed gaseous distension of stomach and intestines (possibly a postmortem alteration), moderate dilatation of the pelvis of the right kidney and a small spleen. The litter consisted of 13 embryos that appeared normal for their developmental ages.

Group V dam 6923 was found dead on GD 12 during the morning check for viability. This rat had soft feces (GD 7–11) and excess salivation (GD 10–11). Body weight and feed consumption values were unremarkable. The abdominal fur appeared urine stained at the necropsy examination, and there were numerous small black spots on the mucosal surface in the fundic region of the stomach. The litter consisted of 14 embryos that appeared normal for their developmental ages.

Group V dam 6918 was found dead on GD 13 of gestation during the morning check for viability. This rat had soft feces (GD 8), liquid feces (GD 9–12), no feces in the cage pan (GD 10), urine-stained abdominal fur (GD 9–12), red perinasal substance (GD 9–12), red substance on both forelimbs and paws (GD 11–12), feces-stained fur (GD 12), ptosis (GD 12) and was cold to touch (GD 12). It had consumed no feed between GD 9 and 12 and had the lowest body weight value in the group on GD 12. Necropsy revealed gaseous distension of the stomach and intestinal tract, possibly a postmortem alteration. The litter consisted of 15 embryos that appeared small for their developmental ages.

Group V dam 6921 was found dead on GD 16. This rat had soft and/or liquid feces (GD 8–15), no feces (GD 8 and GD 15–16), excess salivation (GD 13), dyspnea (GD 14–16), appeared pale (GD 15–16), cold to touch (GD 16), fur stained with feces (GD 8), fur stained with urine (GD 9–10 and GD 15–16) and a red substance around the nose and on both forelimbs and paws (GD 16). Feed consumption values were reduced on GD 12 to 16 of gestation as compared with other dams in the same dosage group, and its body weight was reduced on GD 15 and 16. Necropsy revealed an irregular surface on the lungs with multiple raised areas, a small thymus, enlarged adrenals, multiple black pinpoint spots and no mucosal folds in the stomach, intestines that were gas-filled and thin-walled, and the cecum also had a thin wall and was filled with green-brown mucous. The litter consisted of 16 fetuses that appeared small for their developmental ages.

B.2. Premature Delivery Unrelated to the Test Substance

Group II dam 6835 prematurely delivered on GD 20. Necropsy revealed a small dark red thymus, a pale liver, an enlarged spleen and red substance in the stomach (presumably the remains of a cannibalized conceptus). The litter consisted of one early resorption and nine late resorptions. Five additional conceptuses were presumed cannibalized because the number of implantation sites exceeded the identified conceptuses by that number. The dam had appeared pale on GD 14 to 20; a red substance was found in the cage pan on GD 14 and 16; there were no feces in the cage pan on GD 16; a red substance was observed on the fur on GD 16; a brown perivaginal substance was observed on GD 19 to 20, and urine-stained abdominal fur was observed on GD 20. Feed consumption values were reduced as compared with other rats in Group II on GD 9 to 12, and the body weight of this rat was reduced on day 15 of gestation and thereafter. This premature delivery was considered unrelated to the test substance because it was not dosage-dependent.

C. Clinical Observations

Clinical Observations are summarized in Table 1. Individual animal data for clinical signs of toxicity are summarized in Table 13.

Significantly increased ($P \leq 0.01$) numbers of Group V rats had abnormal feces (soft feces, liquid feces, or no feces), alopecia on the underside, excess salivation, urine-stained abdominal fur and fecal-stained fur. Other observations, such as a red substance around the nose or on both paws and forelimbs, being cold to touch, ptosis, impaired righting reflex, pale appearance, brown perivaginal substance, red substance in cage pan, and a red substance on the abdominal fur occurred only in the rats that died, as described above. Dyspnea was observed on three days in one Group V rat that died and on two days in another rat in this dosage group. Rales occurred once in each of two Group IV rats and three times in one Group V rat that died; the incidences in the Group IV rats were not considered biologically important because they were isolated events. Other clinical observations (e.g., vocalization, swollen snout) were considered unrelated to the test substance because the incidences were not dosage-dependent.

D. Maternal Body Weights and Gravid Uterine Weights

Maternal body weights are summarized in Figure 1 and Tables 2 and 3. Individual animal data for maternal body weights and uterine weight are summarized in Table 14.

Group V dams lost body weight from GD 6 and 9 and body weight gains on GD 9 to 12, for the entire dosage period (calculated as GD 6 to 16), and for

the cumulative intervals of GD 6 to 20 and GD 0 to 20 were significantly reduced ($P \leq 0.01$). Average body weights in Group V were significantly reduced ($P \leq 0.01$) on GD 9 and thereafter. Gravid uterine weights were comparable among the dosage groups so there were still significant reductions ($P \leq 0.01$) in average maternal body weights and body weight changes in Group V when the body weight values on GD 20 were corrected for gravid uterine weight.

E. Maternal Feed Consumption Values

Maternal feed consumption values are summarized in Table 4. Individual animal data for feed consumption values are summarized in Table 15.

Maternal feed consumption values (g/day) were significantly reduced ($P \leq 0.01$) in Group V throughout the dosage period and for the cumulative intervals (calculated as GD 6 to 20 and GD 0 to 20).

Statistically significant decreases ($P \leq 0.05$ to $P \leq 0.01$) were observed in maternal feed consumption values in Groups III or IV or both at several intervals in the dosage and postdosage periods. These events were considered unrelated to the test substance because of the small magnitude of change from the control group value ($< 8\%$) and the fact that the changes in these groups did not occur in a dose-related manner.

F. Necropsy Observations

Necropsy observations are summarized in Table 5. Individual animal data for necropsy observations are summarized in Table 16.

Gross lesions were largely limited to rats that died during the study. These gross lesions were described previously. Gross lesions in rats that survived until scheduled sacrifice included a dark red-brown area in the lung and no apparent thymus tissue in one Group V rat and moderate dilation of the kidney in one Group IV rat.

G. Caesarean-Sectioning and Litter Observations

Caesarean-sectioning and litter observations are summarized in Tables 6 and 7. Individual animal data for Caesarean-sectioning and litter observations are summarized in Tables 17 through 19.

There were 24 or 25 pregnant rats in each group. Caesarean-sectioning observations were based on the 25, 23, 24, 24 and 21 pregnant rats in the 0, 10, 30, 100 and 170 mg/kg/day groups, respectively, that survived until scheduled sacrifice.

Dosages of Hyamine® 1622 as high as 170 mg/kg/day did not affect Caesarean-sectioning observations. The litter averages for corpora lutea, implantations, litter size, live and dead fetuses, early and late resorptions, percent dead or resorbed conceptuses per litter, percent male fetuses per litter and live fetal body weights were similar among the groups and did not significantly differ. One Group II litter (6830) included a dead fetus, and two Group IV litters (6891 and 6895) each included one late resorption; these events were not dosage-dependent and were considered unrelated to the test substance.

H. Fetal Alterations

Fetal alterations are summarized in Tables 8 through 12. Individual animal data for fetal alterations are summarized in Table 20.

Fetal alterations were defined as: 1) malformations (irreversible changes that occur at low incidences in this species and strain); and 2) variations (common findings in this species/strain, and reversible delays or accelerations in development). Litter averages were calculated for specific fetal ossification sites as part of the evaluation of the degree of fetal ossification.

H.1. Summary of Fetal Alterations

Fetal evaluations were based on the number of live fetuses delivered on day 20 of gestation by Caesarean-section. The number of live fetuses in each group was 359, 325, 342, 335 and 270 for Groups I through V, respectively. Each fetus was examined for gross external alterations. Of these respective fetuses, 175, 155, 165, 162 and 131 were examined for soft tissue alterations and 184, 170, 177, 173 and 139 were examined for skeletal alterations and fetal ossification site averages.

A small number of fetal alterations (malformations and variations) were observed in each group, including the vehicle control. These were considered unrelated to the test substance because: 1) the incidence values, including those that were statistically significantly different from control group values, were within the ranges observed historically at the Testing Facility; 2) the incidence values were not dosage-dependent; and/or 3) alterations that occurred only in the high dosage group were present in only one fetus and occurred at incidences that were not statistically significant.

When the incidences of fetal malformations and variations were combined, statistically significant increases ($P \leq 0.05$ to $P \leq 0.01$) in fetal alterations occurred in Groups III and IV. Group III also had a significantly increased ($P \leq 0.01$) incidence of fetal variations. These observations reflect statistically significant increases in the fetal incidences of small reversible delays in ossification of the

sternum (incompletely ossified and/or not ossified sternebrae); these observations were considered unrelated to the test substance because: 1) the values were not dosage-dependent; 2) the litter incidence values, the more relevant parameter, did not significantly differ; and 3) the litter and fetal incidence values were within the ranges observed historically^a at the Testing Facility.

All fetal alterations that occurred in this study are described in the following information.

H.2. Fetal Gross External Alterations

H.2.a. Malformations

One control group fetus (6801-1) had exencephaly. Skeletal examination revealed absence of the frontal and parietal bones, a short maxillae, premaxillae and nasal bones and an incompletely ossified basisphenoid bone in the skull, a bifid centrum in the 11th thoracic vertebra, and an unossified 1st sternal centra. Another control group fetus (6815-18) had cleft palate and micrognathia; no additional alterations were revealed in the soft tissue examination of this fetus.

One Group II fetus and two Group IV fetuses had depressed left eye bulges. Soft tissue examination of the Group II fetus (6837-6) and one Group IV fetus (6890-10) revealed microphthalmia. Skeletal examination of the other Group IV fetus (6885-6) revealed a small left eye socket. Other alterations included a cervical rib at the left side of the 7th cervical vertebra, incomplete ossification of the 1st sternal centra and an incompletely ossified pubis bone. One Group V fetus (6920-7) had no tail. Skeletal examination of this fetus revealed only two sacral vertebrae and no caudal vertebrae.

No other gross external fetal malformations occurred in this study.

H.3. Fetal Soft Tissue Alterations

H.3.a. Malformations

Microphthalmia occurred in two fetuses (Group II fetus, 6837-6; Group IV fetus, 6890-10). One control group fetus (6815-18) had cleft palate, as described

a. See APPENDIX 6 (HISTORICAL CONTROL DATA).

previously. Malformations of the blood vessels were observed in two fetuses: the innominate vessel was absent in another control group fetus (6824-4), and the common carotid originated from the innominate in one Group II fetus (6848-10). No fetal soft tissue malformations occurred in the Group V fetuses in this study.

H.3.b. Variations

Group III fetus (6866-13) had a variation in the umbilical artery as its only alteration: the artery descended to the left of the urinary bladder. No other soft tissue variations occurred in these fetuses.

H.4. Fetal Skeletal Alterations

H.4.a. Malformations

As described previously, the control group fetus (6801-1) with exencephaly had malformations of the skull that included absence of the frontal and parietal bones, short nasal, maxillae and premaxillae bones, and incomplete ossification of the basisphenoid bone. In addition, this fetus had variations in the ossification of thoracic and sternal centra. The Group IV fetus 6885-6 with a depressed eye bulge had a small eye socket and several skeletal variations: a cervical rib and incompletely ossified sternal centrum and pubis bone. The reduction in the number of sacral vertebrae and the absence of caudal vertebrae in the Group V fetus (6920-7) without a tail have also been described.

One Group II fetus (6846-9) and one Group IV fetus (6895-3) had thoracic hemivertebra. The Group IV fetus also had fused centra in the 7th and 8th thoracic vertebrae and bifid centra in the 2nd, 6th, 7th, 8th and 10th thoracic vertebrae.

H.4.b. Variations

Two Group I fetuses (6807-5; 6808-9), one Group II fetus (6849-13), four Group III fetuses (6853-7,-9; 6861-3,-14), and two Group IV fetuses (6890-7; 6885-6) had a cervical rib at the 7th cervical vertebra. The Group II fetus also had an incompletely ossified first sternal centrum, and the externally malformed Group IV fetus 6885-6 had other skeletal malformations and variations that have been described previously.

Three Group I fetuses (6801-1; 6808-13; 6825-5), one Group II fetus (6844-16), five Group III fetuses (6865-13; 6867-9; 6872-14; 6873-5,-7), two Group IV fetuses (6886-5; 6895-3) and two Group V fetuses (6903-3; 6924-15) had one or more bifid thoracic centra. The Group I fetus 6801-1 and the

Group IV fetus 6895-3 had skeletal malformations and other skeletal variations that were described previously.

Two Group I fetuses (6805-5; 6810-5), three Group II fetuses (6834-1,-3,-13) and one Group III fetus (6862-5) had incompletely ossified arches in the lumbar vertebrae, and one Group IV fetus (6894-7) had unilateral ossification of a lumbar centrum. One of the Group I fetuses (6805-5) had wavy ribs, incompletely ossified ribs and an incompletely ossified pubis. Two of the Group II fetuses (6834-1,-3) also had wavy ribs, incompletely ossified ribs and/or incompletely ossified pubes or ischium or both. The third fetus in this group (6834-13) had an incompletely ossified sternal centrum. The Group III fetus 6862-5 also had an incompletely ossified sternal centrum and ischium.

Wavy ribs occurred in six Group I fetuses (6805-5; 6810-3,-7,-11 6818-3; 6819-1), four Group II fetuses (6831-3; 6834-1,-9; 6836-2), five Group III fetuses (6865-3,-5,-7,-11; 6870-10) and five Group V fetuses (6915-3,-10; 6916-12; 6922-7,-9). Of these fetuses, three in each of Groups I (6805-5; 6810-3,-11), II (6831-3; 6834-1,-9) and III (6865-3,-5,-7), and two fetuses in Group V (6915-10; 6922-9) had other skeletal alterations that included incompletely ossified (hypoplastic) ribs. Two other Group I fetuses (6818-3; 6819-1) and one other Group V fetus (6915-3) had additional skeletal alterations that did not include hypoplastic ribs.

Delayed sternal ossification (incompletely ossified and/or not ossified sternbrae) occurred in 2, 9 ($P \leq 0.01$), 10 ($P \leq 0.01$), 3 and 8 ($P \leq 0.01$) fetuses from 2, 5, 5, 3 and 5 litters in Groups I through V, respectively. Incompletely ossified sternal centra occurred in 1, 9 ($P \leq 0.01$), 8 ($P \leq 0.01$), 2 and 3 fetuses from 1, 5, 5, 2 and 3 litters in Groups I through V, respectively. Not ossified sternal centra occurred in 1, 0, 2, 1 and 5 ($P \leq 0.01$) fetuses from 1, 0, 1, 1 and 3 litters from Groups I through V, respectively. The statistically significant increases in the incidences of these alteration in Groups II, III and V were considered unrelated to the test substance because: 1) the fetal incidence values were not dosage-dependent in two of the three categories; 2) the litter incidence values, the more relevant parameter, did not significantly differ; and/or 3) the litter and fetal values were within the ranges observed historically^a at the Testing Facility.

Variations in the ossification of the pelvis were comparable among the five dosage groups: incompletely ossified pubes and ischia occurred in 10, 7, 10, 4 and 7 fetuses from 5, 4, 5, 4 and 3 litters in Groups I through IV, respectively.

a. See APPENDIX 6 (HISTORICAL CONTROL DATA).

H.4.c. Fetal Ossification Site Averages

Analyses of the average numbers of fetal ossification sites per litter did not reveal biologically important or statistically significant differences among the five dosage groups. Ossification of the hyoid, vertebrae (cervical, thoracic, lumbar, sacral and caudal), ribs, sternum (manubrium, sternal centers and xiphoid), forelimbs (carpals, metacarpals and phalanges) and hindlimbs (tarsals, metatarsals and phalanges) occurred at similar incidences in litters in all dosage groups.

V. CONCLUSION

Hyamine® 1622 was not a developmental toxicant in this study. Based upon the data collected in this study, the maternal no-observable-adverse-effect-level (NOAEL) and the developmental NOAEL for the test substance are 100 mg/kg/day and greater than 170 mg/kg/day, respectively.

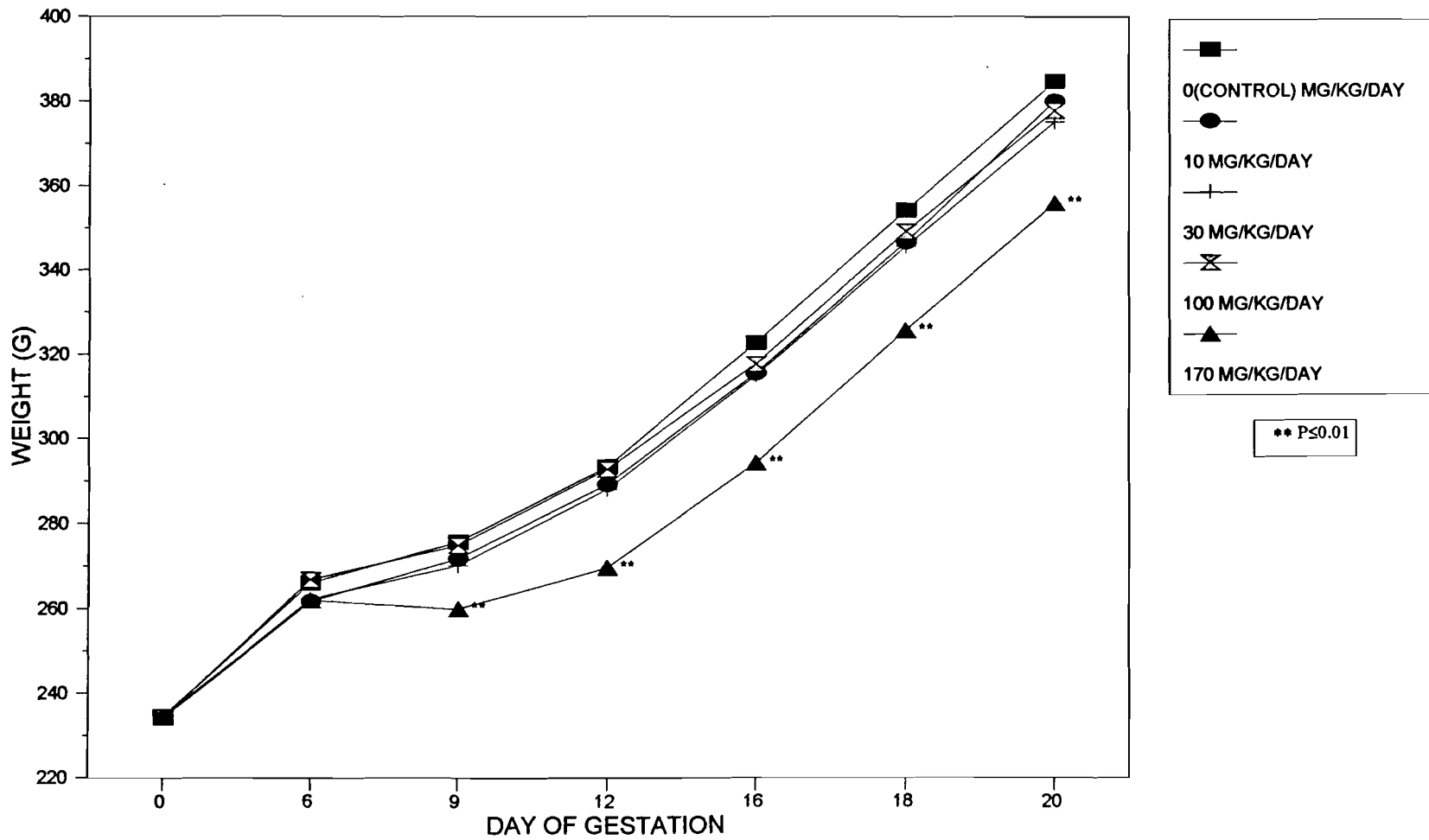
REFERENCES

1. U.S. Environmental Protection Agency (1984). *Pesticide Assessment Guidelines*. Subdivision F - Hazard Evaluation: Human and Domestic Animals, November, 1984 (Revised Edition).
2. U.S. Environmental Protection Agency. Federal Insecticide, Fungicide and Rodenticide Act (FIFRA); Good Laboratory Practice Standards; Final Rule. 40 CFR Part 160.
3. Christian, M.S. and Voytek, P.E. (1982). *In Vivo Reproductive and Mutagenicity Tests*. Environmental Protection Agency, Washington, D.C. National Technical Information Service, U.S. Department of Commerce, Springfield, VA 22161.
4. Christian, M.S. (1984). Reproductive toxicity and teratology evaluations of naltrexone (Proceedings of Naltrexone Symposium, New York Academy of Sciences, November 7, 1983), J. Clin. Psychiat. 45(9):7-10.
5. Lang, P.L. (1988). *Embryo and Fetal Developmental Toxicity (Teratology) Control Data in the Charles River Crl:CD®BR Rat*. Charles River Laboratories, Inc., Wilmington, MA 01887-0630. (Data base provided by Argus Research Laboratories, Inc.)
6. U.S. Department of Health and Human Services (1985). *Guide for the Care and Use of Laboratory Animals*. Prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, Commission on Life Sciences, National Research Council. Public Health Service, National Institutes of Health, NIH Publication No. 86-23.
7. Salewski, E. (1964). Färbemethode zum makroskopischen Nachweis von Implantationsstellen am Uterus der Ratte. Arch. Pathol. Exp. Pharmacol. 247:367.
8. Staples, R.E. (1974). Detection of visceral alterations in mammalian fetuses. Teratology 9(3):A37-38.
9. Modification of method of Staples, R.E. and Schnell, V.L. (1963). Refinement in rapid clearing technique in the KOH-alizarin red S method for fetal bone. Stain Technol. 39:61-63.

10. Snedecor, G.W. and Cochran, W.G. (1967). Variance test for homogeneity of the binomial distribution. *Statistical Methods*, 6th Edition, Iowa State University Press, Ames, pp. 240-241.
11. Sokal, R.R. and Rohlf, F.J. (1969). Bartlett's test of homogeneity of variances. *Biometry*, W.H. Freeman and Co., San Francisco, pp. 370-371.
12. Snedecor, G.W. and Cochran, W.G. (1967). Analysis of Variance. *Statistical Methods*, 6th Edition, Iowa State University Press, Ames, pp. 258-275.
13. Dunnett, C.W. (1955). A multiple comparison procedure for comparing several treatments with a control. *J. Amer. Stat. Assoc.* 50:1096-1129.
14. Sokal, R.R. and Rohlf, F.J. (1969). Kruskal-Wallis Test. *Biometry*, W.H. Freeman and Co., San Francisco, pp. 388-389.
15. Dunn, O.J. (1964). Multiple comparisons using rank sums. *Technometrics* 6(3):241-252.
16. Siegel, S. (1956). *Nonparametric Statistics for the Behavioral Sciences*, McGraw-Hill, New York, pp. 96-104.

MATERNAL BODY WEIGHTS

FIGURE 1



PROTOCOL 720-002: DEVELOPMENTAL TOXICITY STUDY OF HYAMINE® 1622 IN RATS

TABLE 1 (PAGE 1): CLINICAL OBSERVATIONS - SUMMARY

DOSAGE GROUP DOSAGE (MG/KG/DAY) ^a	I 0 (VEHICLE)	II 10	III 30	IV 100	V 170
MAXIMUM POSSIBLE INCIDENCE	375/ 25	375/ 25	375/ 25	375/ 25	347/ 25
MORTALITY	0	1	0	0	4**
FOUND DEAD	0	0	0	0	4**b,c,d,e
SACRIFICED; DAM IN THE PROCESS OF DELIVERY	0	1f	0	0	0
ABNORMAL FECES: TOTAL	0/ 0	1/ 1	0/ 0	0/ 0	132/ 24**
SOFT FECES	0/ 0	0/ 0	0/ 0	0/ 0	117/ 24**b,c,d,e
NO FECES	0/ 0	1/ 1f	0/ 0	0/ 0	12/ 7**b,c,d
LIQUID FECES	0/ 0	0/ 0	0/ 0	0/ 0	15/ 6**b,c,d
LOCALIZED ALOPECIA: TOTAL	0/ 0	21/ 2	0/ 0	6/ 1	69/ 7**
UNDERSIDE	0/ 0	12/ 1	0/ 0	6/ 1	54/ 6**
LIMBS	0/ 0	21/ 2	0/ 0	0/ 0	22/ 2
BACK	0/ 0	0/ 0	0/ 0	0/ 0	9/ 1
EXCESS SALIVATION	0/ 0	0/ 0	0/ 0	0/ 0	10/ 6**b,d,e
URINE-STAINED ABDOMINAL FUR	0/ 0	1/ 1f	0/ 0	0/ 0	19/ 5**b,c,d
FECES-STAINED FUR	0/ 0	0/ 0	0/ 0	0/ 0	4/ 3**c,d
RED PERINASAL SUBSTANCE	0/ 0	0/ 0	0/ 0	0/ 0	8/ 3**b,c,d
FORELIMBS AND PAWS: BILATERAL, RED SUBSTANCE	0/ 0	0/ 0	0/ 0	0/ 0	6/ 3**b,c,d
COLD TO TOUCH	0/ 0	0/ 0	0/ 0	0/ 0	4/ 3**b,c,d

STATISTICAL ANALYSES OF CLINICAL OBSERVATION DATA WERE RESTRICTED TO THE NUMBER OF RATS WITH OBSERVATIONS.
 MAXIMUM POSSIBLE INCIDENCE = (DAYS x RATS)/NUMBER OF RATS EXAMINED PER GROUP ON DAYS 6 THROUGH 20 OF PRESUMED GESTATION.

N/N = TOTAL NUMBER OF OBSERVATIONS/NUMBER OF RATS WITH OBSERVATION.

a. Dosage occurred on day 6 through 15 of presumed gestation.

b. Rat 6910 was found dead on day 11 of gestation

c. Rat 6918 was found dead on day 13 of gestation

d. Rat 6921 was found dead on day 16 of gestation

e. Rat 6923 was found dead on day 12 of gestation

f. Rat 6835 was sacrificed on day 20 of gestation; dam was in the process of delivery.

** Significantly different from the vehicle control group value ($P \leq 0.01$).

PROTOCOL 720-002: DEVELOPMENTAL TOXICITY STUDY OF HYAMINE® 1622 IN RATS

TABLE 1 (PAGE 2): CLINICAL OBSERVATIONS - SUMMARY

DOSAGE GROUP DOSAGE (MG/KG/DAY) ^a	I 0 (VEHICLE)	II 10	III 30	IV 100	V 170
MAXIMUM POSSIBLE INCIDENCE	375/ 25	375/ 25	375/ 25	375/ 25	347/ 25
MORTALITY	0	1	0	0	4**
FOUND DEAD	0	0	0	0	4**b,c,d,e
SACRIFICED; DAM IN THE PROCESS OF DELIVERY	0	1f	0	0	0
DYSPNEA	0/ 0	0/ 0	0/ 0	0/ 0	5/ 2d
RALES	0/ 0	0/ 0	0/ 0	2/ 2	3/ 1b
PTOSIS	0/ 0	0/ 0	0/ 0	0/ 0	1/ 1c
IMPAIRED RIGHTING REFLEX	0/ 0	0/ 0	0/ 0	0/ 0	1/ 1b
VOCALIZATION	0/ 0	0/ 0	0/ 0	0/ 0	1/ 1
SWOLLEN SNOUT	0/ 0	0/ 0	1/ 1	0/ 0	0/ 0
PALE APPEARANCE	0/ 0	7/ 1f	0/ 0	0/ 0	2/ 1d
BROWN PERIVAGINAL SUBSTANCE	0/ 0	2/ 1f	0/ 0	0/ 0	0/ 0
RED SUBSTANCE IN CAGE PAN	0/ 0	2/ 1f	0/ 0	0/ 0	0/ 0
ABDOMINAL FUR: RED SUBSTANCE	0/ 0	1/ 1f	0/ 0	0/ 0	0/ 0

STATISTICAL ANALYSES OF CLINICAL OBSERVATION DATA WERE RESTRICTED TO THE NUMBER OF RATS WITH OBSERVATIONS.
MAXIMUM POSSIBLE INCIDENCE = (DAYS x RATS)/NUMBER OF RATS EXAMINED PER GROUP ON DAYS 6 THROUGH 20 OF PRESUMED GESTATION.

N/N = TOTAL NUMBER OF OBSERVATIONS/NUMBER OF RATS WITH OBSERVATION.

a. Dosage occurred on day 6 through 15 of presumed gestation.

b. Rat 6910 was found dead on day 11 of gestation

c. Rat 6918 was found dead on day 13 of gestation

d. Rat 6921 was found dead on day 16 of gestation

e. Rat 6923 was found dead on day 12 of gestation

f. Rat 6835 was sacrificed on day 20 of gestation; dam was in the process of delivery.

** Significantly different from the vehicle control group value ($P \leq 0.01$).

PROTOCOL 720-002: DEVELOPMENTAL TOXICITY STUDY OF HYAMINE® 1622 IN RATS

TABLE 2 (PAGE 1): MATERNAL BODY WEIGHTS - SUMMARY

DOSAGE GROUP DOSAGE (MG/KG/DAY) ^a		I 0 (VEHICLE)	II 10	III 30	IV 100	V 170
RATS TESTED	N	25	25	25	25	25
PREGNANT	N	25	24	24	24	25
MATERNAL BODY WEIGHT (G)						
DAY 0	MEAN±S.D.	234.5 ± 10.0	234.1 ± 10.7	234.4 ± 9.8	234.3 ± 9.8	234.6 ± 9.8
DAY 6	MEAN±S.D.	266.0 ± 12.8	261.6 ± 11.8	262.1 ± 14.1	266.8 ± 14.1	261.9 ± 7.7
DAY 9	MEAN±S.D.	275.7 ± 13.8	271.7 ± 12.9	270.2 ± 18.5	274.9 ± 16.1	259.9 ± 17.3**
DAY 12	MEAN±S.D.	293.3 ± 16.6	289.2 ± 13.3	288.1 ± 20.3	292.8 ± 18.0	269.6 ± 26.8**
DAY 16	MEAN±S.D.	322.7 ± 18.6	315.5 ± 20.5	315.0 ± 20.8	317.7 ± 18.9	294.3 ± 27.2** [23]b
DAY 18	MEAN±S.D.	354.0 ± 17.9	346.5 ± 24.5	345.5 ± 22.8	349.1 ± 19.2	325.6 ± 21.1** [22]b
DAY 20	MEAN±S.D.	384.6 ± 22.0	380.0 ± 18.4 [23]b	375.1 ± 24.3	377.8 ± 19.7	355.7 ± 23.2** [21]b
GRAVID UTERINE WEIGHT (G)	MEAN±S.D.	79.53 ± 10.31	77.40 ± 10.45 [23]b	75.51 ± 8.42 [23]c	73.72 ± 8.57	67.99 ± 18.90 [21]b
DAY 20C d	MEAN±S.D.	305.1 ± 17.4	302.6 ± 13.9 [23]b	297.7 ± 20.2 [23]c	304.1 ± 21.5	287.7 ± 10.4** [21]b

DAY = DAY OF GESTATION

[] = NUMBER OF VALUES AVERAGED

a. Dosage occurred on days 6 through 15 of gestation.

b. Excludes values for rats that died or were sacrificed early.

c. Excludes a value that was not recorded.

d. 20C = Corrected maternal body weight (day 20 of gestation body weight minus the gravid uterine weight).

** Significantly different from the vehicle control group value ($P \leq 0.01$).

PROTOCOL 720-002: DEVELOPMENTAL TOXICITY STUDY OF HYAMINE® 1622 IN RATS

TABLE 3 (PAGE 1): MATERNAL BODY WEIGHT CHANGES - SUMMARY

DOSAGE GROUP DOSAGE (MG/KG/DAY) ^a		I 0 (VEHICLE)	II 10	III 30	IV 100	V 170
RATS TESTED	N	25	25	25	25	25
PREGNANT	N	25	24	24	24	25
MATERNAL BODY WEIGHT CHANGE (G)						
DAYS 0 - 6	MEAN±S.D.	+31.5 ± 7.2	+27.5 ± 7.8	+27.7 ± 7.4	+32.4 ± 9.9	+27.2 ± 6.7
DAYS 6 - 9	MEAN±S.D.	+9.7 ± 6.1	+10.1 ± 5.7	+8.1 ± 8.1	+8.2 ± 5.7	-2.0 ± 17.4*
DAYS 9 - 12	MEAN±S.D.	+17.6 ± 5.3	+17.5 ± 6.4	+17.9 ± 4.7	+17.8 ± 7.1	+7.5 ± 16.0** [23]b
DAYS 12 - 16	MEAN±S.D.	+29.4 ± 6.8	+26.3 ± 11.6	+26.9 ± 6.7	+25.0 ± 5.8	+20.9 ± 22.4 [22]b
DAYS 6 - 16	MEAN±S.D.	+56.7 ± 10.9	+53.9 ± 17.4	+52.9 ± 9.8	+51.0 ± 9.2	+33.4 ± 28.9** [22]b
DAYS 16 - 18	MEAN±S.D.	+31.3 ± 4.4	+31.0 ± 7.5	+30.5 ± 5.5	+31.4 ± 4.3	+27.3 ± 9.4 [21]b
DAYS 18 - 20	MEAN±S.D.	+30.6 ± 6.1	+29.8 ± 5.0 [23]b	+29.5 ± 4.5	+28.7 ± 4.5	+30.1 ± 8.8 [21]b
DAYS 16 - 20	MEAN±S.D.	+61.9 ± 7.5	+61.6 ± 8.7 [23]b	+60.0 ± 8.1	+60.1 ± 6.6	+57.4 ± 15.2 [21]b
DAYS 6 - 20	MEAN±S.D.	+118.6 ± 15.5	+118.7 ± 12.9 [23]b	+113.0 ± 13.0	+111.0 ± 9.4	+95.3 ± 24.4** [21]b
DAYS 0 - 20	MEAN±S.D.	+150.1 ± 18.2	+146.5 ± 16.4 [23]b	+140.7 ± 17.8	+143.4 ± 16.3	+123.7 ± 25.1** [21]b
DAYS 6 - 20C c	MEAN±S.D.	+39.1 ± 9.5	+41.3 ± 7.4 [23]b	+36.3 ± 10.0 [23]d	+37.3 ± 10.4	+27.3 ± 11.5** [21]b
DAYS 0 - 20C c	MEAN±S.D.	+70.5 ± 13.0	+69.1 ± 12.6 [23]b	+63.5 ± 14.2 [23]d	+69.7 ± 18.0	+55.7 ± 11.9** [21]b

DAYS = DAYS OF GESTATION

[] = NUMBER OF VALUES AVERAGED

a. Dosage occurred on days 6 through 15 of gestation.

b. Excludes values for rats that died or were sacrificed early.

c. 20C = Corrected maternal body weight (day 20 of gestation body weight minus the gravid uterine weight).

d. Excludes values that were not recorded.

* Significantly different from the vehicle control group value (P<0.05).

** Significantly different from the vehicle control group value (P<0.01).

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TABLE 4 (PAGE 1): MATERNAL FEED CONSUMPTION VALUES - SUMMARY

DOSAGE GROUP DOSAGE (MG/KG/DAY) ^a		I 0 (VEHICLE)	II 10	III 30	IV 100	V 170
RATS TESTED	N	25	25	25	25	25
PREGNANT	N	25	24	24	24	25
MATERNAL FEED CONSUMPTION (G/DAY)						
DAYS 0 - 6	MEAN±S.D.	21.6 ± 2.2	20.8 ± 2.5	21.0 ± 2.2	22.7 ± 2.4	21.4 ± 1.6
DAYS 6 - 9	MEAN±S.D.	22.7 ± 2.7	22.1 ± 3.0	20.8 ± 3.5	21.4 ± 2.8	15.1 ± 4.3**
DAYS 9 - 12	MEAN±S.D.	24.5 ± 2.2	23.3 ± 3.3	22.8 ± 2.6*	23.6 ± 3.6	[24] ^b 16.8 ± 6.0**
DAYS 12 - 16	MEAN±S.D.	25.6 ± 2.1	24.2 ± 4.3	23.8 ± 2.4* [23] ^b	23.6 ± 2.6**	20.2 ± 5.3** [22] ^c
DAYS 6 - 16	MEAN±S.D.	24.4 ± 2.1	23.3 ± 3.3	22.6 ± 2.5 [23] ^b	23.0 ± 2.8	18.0 ± 3.6** [22] ^c
DAYS 16 - 18	MEAN±S.D.	28.2 ± 1.9	27.2 ± 4.2	27.0 ± 2.9	28.5 ± 2.7	29.4 ± 2.3 [21] ^c
DAYS 18 - 20	MEAN±S.D.	27.3 ± 2.0	26.4 ± 2.2 [23] ^c	25.6 ± 2.4*	27.1 ± 2.8	28.4 ± 2.5 [21] ^c
DAYS 16 - 20	MEAN±S.D.	27.7 ± 1.7	27.2 ± 2.1 [23] ^c	26.3 ± 2.6	27.8 ± 2.7	28.9 ± 2.0 [21] ^c
DAYS 6 - 20	MEAN±S.D.	25.4 ± 1.9	24.7 ± 2.0 [23] ^c	23.6 ± 2.4*	24.3 ± 2.6	21.5 ± 2.3** [21] ^c
DAYS 0 - 20	MEAN±S.D.	24.2 ± 1.7	23.6 ± 2.0 [23] ^c	22.9 ± 2.2*	23.9 ± 2.5	21.5 ± 1.8** [21] ^c

DAYS = DAYS OF GESTATION

[] = NUMBER OF VALUES AVERAGED

a. Dosage occurred on days 6 through 15 of gestation.

b. Excludes values that were associated with spillage or soiled feed.

c. Excludes values for rats that died or were sacrificed early.

* Significantly different from the vehicle control group value ($P \leq 0.05$).

** Significantly different from the vehicle control group value ($P \leq 0.01$).

PROTOCOL 720-002: DEVELOPMENTAL TOXICITY STUDY OF HYAMINE® 1622 IN RATS

TABLE 5 (PAGE 1): NECROPSY OBSERVATIONS - SUMMARY

DOSAGE GROUP DOSAGE (MG/KG/DAY) ^a		I 0 (VEHICLE)	II 10	III 30	IV 100	V 170
RATS EXAMINED	N	25	25	25	25	25
MORTALITY	N	0	1	0	0	4**
FOUND DEAD	N	0	0	0	0	4** ^{b,c,d,e}
SACRIFICED; DAM IN THE PROCESS OF DELIVERY	N	0	1 ^f	0	0	0
APPEARED NORMAL	N	25	24	25	24	20**
LUNGS:						
LEFT APICAL, CRANIAL PORTION, DARK RED-BROWN	N	0	0	0	0	1
ENTIRE SURFACE, IRREGULAR WITH MULTIPLE RAISED AREAS	N	0	0	0	0	1 ^d
STOMACH:						
BLACK SPOTS	N	0	0	0	0	2 ^{d,e}
NO MUCOSAL FOLDS PRESENT	N	0	0	0	0	1 ^d
RED SUBSTANCE	N	0	1 ^f	0	0	0
STOMACH AND/OR INTESTINAL TRACT:						
GAS-FILLED	N	0	0	0	0	2 ^{b,c}
THIN-WALLED AND GAS-FILLED	N	0	0	0	0	1 ^d
CECUM:						
FILLED WITH GREEN-BROWN MUCOUS; THIN-WALLED	N	0	0	0	0	1 ^d
KIDNEYS:						
PELVIS, MODERATE DILATION	N	0	0	0	1	1 ^b

a. Dosage occurred on days 6 through 15 of presumed gestation.

b. Dam 6910 was found dead on day 11 of gestation.

c. Dam 6918 was found dead on day 13 of gestation.

d. Dam 6921 was found dead on day 16 of gestation.

e. Dam 6923 was found dead on day 12 of gestation.

f. Rat 6835 was sacrificed on day 20 of gestation; dam was in the process of delivery.

** Significantly different from the vehicle control group value ($P \leq 0.01$).

ARGUS 720-002

PROTOCOL 720-002: DEVELOPMENTAL TOXICITY STUDY OF HYAMINE® 1622 IN RATS

TABLE 5 (PAGE 2): NECROPSY OBSERVATIONS - SUMMARY

DOSAGE GROUP DOSAGE (MG/KG/DAY) ^a		I 0 (VEHICLE)	II 10	III 30	IV 100	V 170
RATS EXAMINED	N	25	25	25	25	25
MORTALITY	N	0	1	0	0	4**
FOUND DEAD	N	0	0	0	0	4**b,c,d,e
SACRIFICED; DAM IN THE PROCESS OF DELIVERY	N	0	1f	0	0	0
ADRENALS:						
BILATERAL, LARGE	N	0	0	0	0	1d
THYMUS:						
ABSENT	N	0	0	0	0	1
SMALL AND/OR DARK RED	N	0	1f	0	0	1d
SPLEEN:						
SMALL	N	0	0	0	0	1b
LARGE	N	0	1f	0	0	0
LIVER:						
PALE	N	0	1f	0	0	0
EXTERNAL OBSERVATION:						
URINE-STAINED ABDOMINAL FUR	N	0	0	0	0	1e

a. Dosage occurred on days 6 through 15 of presumed gestation.

b. Dam 6910 was found dead on day 11 of gestation.

c. Dam 6918 was found dead on day 13 of gestation.

d. Dam 6921 was found dead on day 16 of gestation.

e. Dam 6923 was found dead on day 12 of gestation.

f. Rat 6835 was sacrificed on day 20 of gestation; dam was in the process of delivery.

** Significantly different from the vehicle control group value ($P \leq 0.01$).

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TABLE 6 (PAGE 1): CAESAREAN-SECTIONING OBSERVATIONS - SUMMARY

DOSAGE GROUP DOSAGE (MG/KG/DAY) ^a		I 0 (VEHICLE)	II 10	III 30	IV 100	V 170
RATS TESTED	N	25	25	25	25	25
PREGNANT	N(%)	25(100.0)	24(96.0)	24(96.0)	24(96.0)	25(100.0)
FOUND DEAD	N(%)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	4(16.0)
SACRIFICED; DAM IN THE PROCESS OF DELIVERY	N(%)	0(0.0)	1(4.2)	0(0.0)	0(0.0)	0(0.0)
RATS PREGNANT AND CAESAREAN-SECTIONED ON DAY 20 OF GESTATION	N	25	23	24	24	21
CORPORA LUTEA	MEAN±S.D.	16.8 ± 2.2	17.1 ± 3.1	16.6 ± 2.0	16.8 ± 1.6	16.6 ± 2.9
IMPLANTATIONS	MEAN±S.D.	14.8 ± 1.9	15.0 ± 1.6	15.0 ± 1.5	15.3 ± 1.5	14.0 ± 2.8
LITTER SIZES	MEAN±S.D.	14.4 ± 1.8	14.2 ± 1.9	14.2 ± 1.6	14.0 ± 1.7	12.8 ± 3.4
LIVE FETUSES	N	359	325	342	335	270
	MEAN±S.D.	14.4 ± 1.8	14.1 ± 1.8	14.2 ± 1.6	14.0 ± 1.7	12.8 ± 3.4
DEAD FETUSES	N	0	1	0	0	0
	MEAN±S.D.	0.0 ± 0.0	0.0 ± 0.2	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
RESORPTIONS	MEAN±S.D.	0.5 ± 0.7	0.8 ± 1.0	0.7 ± 0.9	1.3 ± 1.2	1.1 ± 2.0
EARLY RESORPTIONS	N	12	19	17	30	24
	MEAN±S.D.	0.5 ± 0.7	0.8 ± 1.0	0.7 ± 0.9	1.2 ± 1.2	1.1 ± 2.0
LATE RESORPTIONS	N	0	0	0	2	0
	MEAN±S.D.	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.1 ± 0.3	0.0 ± 0.0
DAMS WITH ANY RESORPTIONS	N(%)	9(36.0)	11(47.8)	12(50.0)	17(70.8)	10(47.6)
DAMS WITH ALL CONCEPTUSES DEAD OR RESORBED	N	0	0	0	0	0
DAMS WITH VIABLE FETUSES	N(%)	25(100.0)	23(100.0)	24(100.0)	24(100.0)	21(100.0)

a. Dosage occurred on days 6 through 15 of gestation.

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TABLE 7 (PAGE 1): LITTER OBSERVATIONS (CAESAREAN-DELIVERED FETUSES) - SUMMARY

DOSAGE GROUP DOSAGE (MG/KG/DAY) ^a		I 0 (VEHICLE)	II 10	III 30	IV 100	V 170
LITTERS WITH ONE OR MORE LIVE FETUSES	N	25	23	24	24	21
IMPLANTATIONS	MEAN±S.D.	14.8 ± 1.9	15.0 ± 1.6	15.0 ± 1.5	15.3 ± 1.5	14.0 ± 2.8
LIVE FETUSES	N	359	325	342	335	270
	MEAN±S.D.	14.4 ± 1.8	14.1 ± 1.8	14.2 ± 1.6	14.0 ± 1.7	12.8 ± 3.4
LIVE MALE FETUSES	N	169	169	183	166	138
% LIVE MALE FETUSES/LITTER	MEAN±S.D.	47.5 ± 15.7	52.0 ± 11.3	53.9 ± 9.2	49.0 ± 14.0	52.6 ± 11.6
LIVE FETAL BODY WEIGHTS (GRAMS)/LITTER	MEAN±S.D.	3.37 ± 0.21	3.36 ± 0.25	3.31 ± 0.22	3.24 ± 0.27	3.27 ± 0.37
MALE FETUSES	MEAN±S.D.	3.46 ± 0.21	3.46 ± 0.26	3.43 ± 0.22	3.30 ± 0.29	3.34 ± 0.37
FEMALE FETUSES	MEAN±S.D.	3.31 ± 0.21	3.24 ± 0.25	3.18 ± 0.24	3.14 ± 0.29	3.19 ± 0.39
% DEAD OR RESORBED CONCEPTUSES/LITTER	MEAN±S.D.	3.1 ± 4.6	5.8 ± 7.1	4.6 ± 5.9	8.6 ± 8.0	8.2 ± 14.4

a. Dosage occurred on days 6 through 15 of gestation.

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TABLE 8 (PAGE 1): FETAL ALTERATIONS - SUMMARY

DOSAGE GROUP DOSAGE (MG/KG/DAY) ^a		I 0(VEHICLE)	II 10	III 30	IV 100	V 170
LITTERS EVALUATED	N	25	23	24	24	21
FETUSES EVALUATED	N	359	326	342	335	270
LIVE	N	359	325	342	335	270
DEAD	N	0	1b	0	0	0
LITTERS WITH FETUSES WITH ANY ALTERATION OBSERVED	N(%)	11(44.0)	11(47.8)	15(62.5)	8(33.3)	9(42.8)
FETUSES WITH ANY ALTERATION OBSERVED	N(%)	21(5.8)	20(6.2)	31(9.1)**	11(3.3)*	19(7.0)
% FETUSES WITH ANY ALTERATION/LITTER	MEAN±S.D.	5.63 ± 8.28	6.33 ± 10.46	9.00 ± 11.35	3.42 ± 5.80	7.29 ± 10.69
LITTERS WITH FETUSES WITH ANY MALFORMATION OBSERVED	N(%)	3(12.0)	3(13.0)	0(0.0)	3(12.5)	1(4.8)
FETUSES WITH ANY MALFORMA- TION OBSERVED	N(%)	3(0.8)	3(0.9)	0(0.0)	3(0.9)	1(0.4)
% FETUSES WITH ANY MALFORMATION/LITTER	MEAN±S.D.	0.83 ± 2.30	0.87 ± 2.32	0.00 ± 0.00	1.06 ± 2.95	0.34 ± 1.55
LITTERS WITH FETUSES WITH ANY VARIATION OBSERVED	N(%)	10(40.0)	9(39.1)	15(62.5)	8(33.3)	8(38.1)
FETUSES WITH ANY VARIATION OBSERVED	N(%)	19(5.3)	17(5.2)	31(9.1)**	10(3.0)	18(6.7)
% FETUSES WITH ANY VARIATION/LITTER	MEAN±S.D.	5.09 ± 7.70	5.46 ± 10.42	9.00 ± 11.35	3.10 ± 5.29	6.95 ± 10.81

a. Dosage occurred on days 6 through 15 of gestation.

b. Dead fetus was excluded from group averages and statistical analyses. Observations for this conceptus are cited on Table 21.

* Significantly different from the vehicle control group value ($P<0.05$).

** Significantly different from the vehicle control group value ($P<0.01$).

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TABLE 9 (PAGE 1): FETAL GROSS EXTERNAL ALTERATIONS - SUMMARY

DOSAGE GROUP		I	II	III	IV	V
DOSAGE (MG/KG/DAY) ^a		0 (VEHICLE)	10	30	100	170
LITTERS EVALUATED	N	25	23	24	24	21
FETUSES EVALUATED	N	359	326	342	335	270
LIVE	N	359	325	342	335	270
DEAD	N	0	1b	0	0	0
HEAD: EXENCEPHALY (M)						
LITTER INCIDENCE	N(%)	1(4.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)
FETAL INCIDENCE	N(%)	1(0.3)	0(0.0)	0(0.0)	0(0.0)	0(0.0)
EYE: BULGE DEPRESSED (M)						
LITTER INCIDENCE	N(%)	0(0.0)	1(4.3)	0(0.0)	2(8.3)	0(0.0)
FETAL INCIDENCE	N(%)	0(0.0)	1(0.3)	0(0.0)	2(0.6)	0(0.0)
PALATE: CLEFT (M)						
LITTER INCIDENCE	N(%)	1(4.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)
FETAL INCIDENCE	N(%)	1(0.3) ^c	0(0.0)	0(0.0)	0(0.0)	0(0.0)
JAW: MICROGNATHIA (M)						
LITTER INCIDENCE	N(%)	1(4.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)
FETAL INCIDENCE	N(%)	1(0.3) ^c	0(0.0)	0(0.0)	0(0.0)	0(0.0)
TAIL: ABSENT (M)						
LITTER INCIDENCE	N(%)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	1(4.8)
FETAL INCIDENCE	N(%)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	1(0.4)

M = MALFORMATION

a. Dosage occurred on days 6 through 15 of gestation.

b. Dead fetus was excluded from group averages and statistical analyses. Observations for this conceptus are cited on Table 20.

c. Fetus 6815-18 also had other gross external alterations.

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TABLE 10 (PAGE 1): FETAL SOFT TISSUE ALTERATIONS - SUMMARY

DOSAGE GROUP		I	II	III	IV	V
DOSAGE (MG/KG/DAY) ^a		0 (VEHICLE)	10	30	100	170
LITTERS EVALUATED	N	25	23	24	24	21
FETUSES EVALUATED	N	175b	156	165	162	131
LIVE	N	175b	155	165	162	131
DEAD	N	0	1c	0	0	0
EYES: MICROPTHALMIA (M)						
LITTER INCIDENCE	N(%)	0(0.0)	1(4.3)	0(0.0)	1(4.2)	0(0.0)
FETAL INCIDENCE	N(%)	0(0.0)	1(0.6)	0(0.0)	1(0.6)	0(0.0)
PALATE: CLEFT (M)						
LITTER INCIDENCE	N(%)	1(4.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)
FETAL INCIDENCE	N(%)	1(0.6)	0(0.0)	0(0.0)	0(0.0)	0(0.0)
VESSELS: COMMON CAROTID ORIGINATES FROM THE INOMINATE (M)						
LITTER INCIDENCE	N(%)	0(0.0)	1(4.3)	0(0.0)	0(0.0)	0(0.0)
FETAL INCIDENCE	N(%)	0(0.0)	1(0.6)	0(0.0)	0(0.0)	0(0.0)
VESSELS: INOMINATE, ABSENT (M)						
LITTER INCIDENCE	N(%)	1(4.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)
FETAL INCIDENCE	N(%)	1(0.6)	0(0.0)	0(0.0)	0(0.0)	0(0.0)
UMBILICAL ARTERY: DESCENDS TO THE LEFT OF URINARY BLADDER (V)						
LITTER INCIDENCE	N(%)	0(0.0)	0(0.0)	1(4.2)	0(0.0)	0(0.0)
FETAL INCIDENCE	N(%)	0(0.0)	0(0.0)	1(0.6)	0(0.0)	0(0.0)

M = MALFORMATION V = VARIATION

a. Dosage occurred on days 6 through 15 of gestation.

b. Includes values for fetus 6817-10; the head was examined and appeared normal. All other observations were not recorded.

c. Dead fetus was excluded from group averages and statistical analyses. Observations for this conceptus are cited on Table 20.

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TABLE 11 (PAGE 1): FETAL SKELETAL ALTERATIONS - SUMMARY
(See footnotes on the last page of this table.)

DOSAGE GROUP		I	II	III	IV	V
DOSAGE (MG/KG/DAY) ^a		0 (VEHICLE)	10	30	100	170
LITTERS EVALUATED	N	25	23	24	24	21
FETUSES EVALUATED	N	184	170	177	173	139
LIVE	N	184	170	177	173	139
DEAD	N	0	0	0	0	0
SKULL: EYE SOCKET, SMALL (M)						
LITTER INCIDENCE	N(%)	0(0.0)	0(0.0)	0(0.0)	1(4.2)	0(0.0)
FETAL INCIDENCE	N(%)	0(0.0)	0(0.0)	0(0.0)	1(0.6) ^y	0(0.0)
SKULL: NASALS, SHORT (M)						
LITTER INCIDENCE	N(%)	1(4.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)
FETAL INCIDENCE	N(%)	1(0.5) ^b	0(0.0)	0(0.0)	0(0.0)	0(0.0)
SKULL: MAXILLAE AND PREMAXILLAE, SHORT (M)						
LITTER INCIDENCE	N(%)	1(4.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)
FETAL INCIDENCE	N(%)	1(0.5) ^b	0(0.0)	0(0.0)	0(0.0)	0(0.0)
SKULL: BASISPHENOID, INCOMPLETELY OSSIFIED (M)						
LITTER INCIDENCE	N(%)	1(4.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)
FETAL INCIDENCE	N(%)	1(0.5) ^b	0(0.0)	0(0.0)	0(0.0)	0(0.0)
SKULL: FRONTALS, NOT OSSIFIED (M)						
LITTER INCIDENCE	N(%)	1(4.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)
FETAL INCIDENCE	N(%)	1(0.5) ^b	0(0.0)	0(0.0)	0(0.0)	0(0.0)
SKULL: PARIETALS, NOT OSSIFIED (M)						
LITTER INCIDENCE	N(%)	1(4.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)
FETAL INCIDENCE	N(%)	1(0.5) ^b	0(0.0)	0(0.0)	0(0.0)	0(0.0)
CERVICAL VERTEBRAE: CERVICAL RIB AT 7TH CERVICAL VERTEBRA (V)						
LITTER INCIDENCE	N(%)	2(8.0)	1(4.3)	2(8.3)	2(8.3)	0(0.0)
FETAL INCIDENCE	N(%)	2(1.1)	1(0.6) ^q	4(2.2)	2(1.2) ^y	0(0.0)
THORACIC VERTEBRAE: CENTRUM, BIFID (V)						
LITTER INCIDENCE	N(%)	3(12.0)	1(4.3)	4(16.7)	2(8.3)	2(9.5)
FETAL INCIDENCE	N(%)	3(1.6) ^b	1(0.6)	5(2.8)	2(1.2) ^z	2(1.4)

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TABLE 11 (PAGE 2): FETAL SKELETAL ALTERATIONS - SUMMARY
(See footnotes on the last page of this table.)

DOSAGE GROUP DOSAGE (MG/KG/DAY) ^a		I 0 (VEHICLE)	II 10	III 30	IV 100	V 170
LITTERS EVALUATED	N	25	23	24	24	21
FETUSES EVALUATED	N	184	170	177	173	139
LIVE	N	184	170	177	173	139
DEAD	N	0	0	0	0	0
THORACIC VERTEBRAE: HEMIVERTEBRA (M)						
LITTER INCIDENCE	N(%)	0(0.0)	1(4.3)	0(0.0)	1(4.2)	0(0.0)
FETAL INCIDENCE	N(%)	0(0.0)	1(0.6)	0(0.0)	1(0.6)z	0(0.0)
THORACIC VERTEBRAE: CENTRA, FUSED (M)						
LITTER INCIDENCE	N(%)	0(0.0)	0(0.0)	0(0.0)	1(4.2)	0(0.0)
FETAL INCIDENCE	N(%)	0(0.0)	0(0.0)	0(0.0)	1(0.6)z	0(0.0)
LUMBAR VERTEBRAE: ARCH, INCOMPLETELY OSSIFIED (V)						
LITTER INCIDENCE	N(%)	2(8.0)	1(4.3)	1(4.2)	0(0.0)	0(0.0)
FETAL INCIDENCE	N(%)	2(1.1)c	3(1.8)l,m,o	1(0.6)s	0(0.0)	0(0.0)
LUMBAR VERTEBRAE: CENTRUM, UNILATERAL OSSIFICATION (V)						
LITTER INCIDENCE	N(%)	0(0.0)	0(0.0)	0(0.0)	1(4.2)	0(0.0)
FETAL INCIDENCE	N(%)	0(0.0)	0(0.0)	0(0.0)	1(0.6)	0(0.0)
SACRAL VERTEBRAE: 2 PRESENT (M)						
LITTER INCIDENCE	N(%)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	1(4.8)
FETAL INCIDENCE	N(%)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	1(0.7)ee
CAUDAL VERTEBRAE: 0 PRESENT (M)						
LITTER INCIDENCE	N(%)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	1(4.8)
FETAL INCIDENCE	N(%)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	1(0.7)ee
RIBS: WAVY (V)						
LITTER INCIDENCE	N(%)	4(16.0)	3(13.0)	2(8.3)	0(0.0)	3(14.3)
FETAL INCIDENCE	N(%)	6(3.3)c,e,f,h,i	4(2.4)k,l,n	5(2.8)u,v,w	0(0.0)	5(3.6)bb, cc,ff
RIBS: INCOMPLETELY OSSIFIED (HYPOPLASTIC) (V)						
LITTER INCIDENCE	N(%)	2(8.0)	2(8.7)	1(4.2)	0(0.0)	2(9.5)
FETAL INCIDENCE	N(%)	4(2.2)c,d,e,f	4(2.4)k,l,m,n	3(1.7)u,v,w	0(0.0)	2(1.4)cc, ff

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TABLE 11 (PAGE 3): FETAL SKELETAL ALTERATIONS - SUMMARY
(See footnotes on the last page of this table.)

DOSAGE GROUP DOSAGE (MG/KG/DAY) ^a		I 0 (VEHICLE)	II 10	III 30	IV 100	V 170
LITTERS EVALUATED	N	25	23	24	24	21
FETUSES EVALUATED	N	184	170	177	173	139
LIVE	N	184	170	177	173	139
DEAD	N	0	0	0	0	0
STERNAL CENTRA SUMMARIZATION (Includes incompletely and not ossified sternal centra):						
LITTER INCIDENCE	N(%)	2(8.0)	5(21.7)	5(20.8)	3(12.5)	5(23.8)
FETAL INCIDENCE	N(%)	2(1.1)	9(5.3)**	10(5.6)**	3(1.7)	8(5.8)**
STERNAL CENTRA: INCOMPLETELY OSSIFIED (V)						
LITTER INCIDENCE	N(%)	1(4.0)	5(21.7)	5(20.8)	2(8.3)	3(14.3)
FETAL INCIDENCE	N(%)	1(0.5)l	9(5.3)**n,o,p,q	8(4.5)**r,s,t,x	2(1.2)y	3(2.2)
STERNAL CENTRA: NOT OSSIFIED (V)						
LITTER INCIDENCE	N(%)	1(4.0)	0(0.0)	1(4.2)	1(4.2)	3(14.3)
FETAL INCIDENCE	N(%)	1(0.5)b	0(0.0)	2(1.1)	1(0.6)	5(3.6)** dd,gg
PELVIS SUMMARIZATION (Includes incompletely ossified pubes and ischia):						
LITTER INCIDENCE	N(%)	5(20.0)	4(17.4)	5(20.8)	4(16.7)	3(14.3)
FETAL INCIDENCE	N(%)	10(5.4)	7(4.1)	10(5.6)	4(2.3)	7(5.0)
PELVIS: PUBIS, INCOMPLETELY OSSIFIED (V)						
LITTER INCIDENCE	N(%)	5(20.0)	4(17.4)	5(20.8)	4(16.7)	3(14.3)
FETAL INCIDENCE	N(%)	10(5.4)c,d,g,h,i	4(2.4)j,l,p	6(3.4)r,x	4(2.3)y	6(4.3)aa, bb,dd,gg
PELVIS: ISCHIUM, INCOMPLETELY OSSIFIED (V)						
LITTER INCIDENCE	N(%)	3(12.0)	2(8.7)	1(4.2)	0(0.0)	2(9.5)
FETAL INCIDENCE	N(%)	3(1.6)d,g,h	5(2.9)j,l,m,n	5(2.8)r,s,t	0(0.0)	3(2.2)aa, cc,dd

TABLE 11 (PAGE 4): FETAL SKELETAL ALTERATIONS - SUMMARY

FOOTNOTES:

M = MALFORMATION V = VARIATION

- a. Dosage occurred on days 6 through 15 of gestation.
- b. Fetus 6801-1 also had other skeletal alterations.
- c. Fetus 6805-5 also had other skeletal alterations.
- d. Fetus 6805-9 also had other skeletal alterations.
- e. Fetus 6810-3 also had other skeletal alterations.
- f. Fetus 6810-11 also had other skeletal alterations.
- g. Fetus 6815-12 also had other skeletal alterations.
- h. Fetus 6818-3 also had other skeletal alterations.
- i. Fetus 6819-1 also had other skeletal alterations.
- j. Fetus 6829-13 also had other skeletal alterations.
- k. Fetus 6831-3 also had other skeletal alterations.
- l. Fetus 6834-1 also had other skeletal alterations.
- m. Fetus 6834-3 also had other skeletal alterations.
- n. Fetus 6834-9 also had other skeletal alterations.
- o. Fetus 6834-13 also had other skeletal alterations.
- p. Fetus 6836-9 also had other skeletal alterations.
- q. Fetus 6849-13 also had other skeletal alterations.
- r. Fetus 6862-3 also had other skeletal alterations.
- s. Fetus 6862-5 also had other skeletal alterations.
- t. Fetus 6862-14 also had other skeletal alterations.
- u. Fetus 6865-3 also had other skeletal alterations.
- v. Fetus 6865-5 also had other skeletal alterations.
- w. Fetus 6865-7 also had other skeletal alterations.
- x. Fetus 6874-9 also had other skeletal alterations.
- y. Fetus 6885-6 also had other skeletal alterations.
- z. Fetus 6895-3 also had other skeletal alterations.
- aa. Fetus 6912-7 also had other skeletal alterations.
- bb. Fetus 6915-3 also had other skeletal alterations.
- cc. Fetus 6915-10 also had other skeletal alterations.
- dd. Fetus 6915-12 also had other skeletal alterations.
- ee. Fetus 6920-7 also had other skeletal alterations.
- ff. Fetus 6922-9 also had other skeletal alterations.
- gg. Fetus 6925-11 also had other skeletal alterations.
- ** Significantly different from the vehicle control group value ($P \leq 0.01$).

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TABLE 12 (PAGE 1): FETAL OSSIFICATION SITES - CAESAREAN-DELIVERED LIVE FETUSES (DAY 20 OF GESTATION) - SUMMARY

DOSAGE GROUP		I	II	III	IV	V
DOSAGE (MG/KG/DAY) ^a		0 (VEHICLE)	10	30	100	170
LITTERS EXAMINED	N	25	23	24	24	21
FETUSES EXAMINED	N	184	170	177	173	139
OSSIFICATION SITES PER FETUS PER LITTER						
HYOID	MEAN+S.D.	0.81 ± 0.18	0.80 ± 0.24	0.85 ± 0.15	0.86 ± 0.18	0.91 ± 0.13
VERTEBRAE						
CERVICAL	MEAN+S.D.	7.00 ± 0.00	7.00 ± 0.00	7.00 ± 0.00	7.00 ± 0.00	7.00 ± 0.00
THORACIC	MEAN+S.D.	13.01 ± 0.04	13.01 ± 0.03	13.01 ± 0.03	13.02 ± 0.06	13.00 ± 0.00
LUMBAR	MEAN+S.D.	5.98 ± 0.05	5.97 ± 0.07	5.98 ± 0.05	5.98 ± 0.07	5.98 ± 0.05
SACRAL	MEAN+S.D.	3.00 ± 0.00	3.00 ± 0.00	3.00 ± 0.00	3.00 ± 0.00	3.00 ± 0.00
CAUDAL	MEAN+S.D.	4.90 ± 0.51	4.87 ± 0.36	4.88 ± 0.34	4.75 ± 0.52	4.72 ± 0.76
RIBS (PAIRS)	MEAN+S.D.	13.01 ± 0.03	13.01 ± 0.04	13.01 ± 0.03	13.01 ± 0.04	13.00 ± 0.00
STERNUM						
MANUBRIUM	MEAN+S.D.	1.00 ± 0.00	1.00 ± 0.02	1.00 ± 0.00	0.99 ± 0.04	1.00 ± 0.02
STERNAL CENTERS	MEAN+S.D.	3.58 ± 0.32	3.57 ± 0.24	3.58 ± 0.32	3.55 ± 0.36	3.42 ± 0.42
XIPHOID	MEAN+S.D.	0.98 ± 0.06	0.99 ± 0.04	1.00 ± 0.02	0.99 ± 0.04	0.99 ± 0.03
FORELIMB b						
CARPALS	MEAN+S.D.	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
METACARPALS	MEAN+S.D.	3.47 ± 0.35	3.48 ± 0.31	3.45 ± 0.28	3.52 ± 0.29	3.51 ± 0.37
DIGITS	MEAN+S.D.	5.00 ± 0.00	5.00 ± 0.00	5.00 ± 0.00	5.00 ± 0.00	5.00 ± 0.00
PHALANGES	MEAN+S.D.	5.08 ± 0.32	5.04 ± 0.10	5.03 ± 0.06	5.04 ± 0.11	5.08 ± 0.21
HINDLIMB b						
TARSALS	MEAN+S.D.	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
METATARSALS	MEAN+S.D.	3.99 ± 0.04	3.99 ± 0.04	4.00 ± 0.00	3.98 ± 0.06	3.99 ± 0.03
DIGITS	MEAN+S.D.	5.00 ± 0.00	5.00 ± 0.00	5.00 ± 0.00	5.00 ± 0.00	5.00 ± 0.00
PHALANGES	MEAN+S.D.	4.97 ± 0.14	5.00 ± 0.00	4.98 ± 0.12	4.84 ± 0.48	4.86 ± 0.41

a. Dosage occurred on days 6 through 15 of gestation.

b. Calculated as average per limb.

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TABLE 13 (PAGE 1): CLINICAL OBSERVATIONS - INDIVIDUAL DATA

DOSAGE GROUP I		0(VEHICLE) MG/KG/DAY
RAT #	DESCRIPTION	
6801	NO ADVERSE FINDINGS	
6802	NO ADVERSE FINDINGS	
6803	NO ADVERSE FINDINGS	
6804	NO ADVERSE FINDINGS	
6805	NO ADVERSE FINDINGS	
6806	NO ADVERSE FINDINGS	
6807	NO ADVERSE FINDINGS	
6808	NO ADVERSE FINDINGS	
6809	NO ADVERSE FINDINGS	
6810	NO ADVERSE FINDINGS	
6811	NO ADVERSE FINDINGS	
6812	NO ADVERSE FINDINGS	
6813	NO ADVERSE FINDINGS	
6814	NO ADVERSE FINDINGS	
6815	NO ADVERSE FINDINGS	
6816	NO ADVERSE FINDINGS	
6817	NO ADVERSE FINDINGS	
6818	NO ADVERSE FINDINGS	
6819	NO ADVERSE FINDINGS	
6820	NO ADVERSE FINDINGS	
6821	NO ADVERSE FINDINGS	
6822	NO ADVERSE FINDINGS	
6823	NO ADVERSE FINDINGS	
6824	NO ADVERSE FINDINGS	
6825	NO ADVERSE FINDINGS	

DG = DAY OF PRESUMED GESTATION

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TABLE 13 (PAGE 2): CLINICAL OBSERVATIONS - INDIVIDUAL DATA

DOSAGE GROUP II		10 MG/KG/DAY
RAT #		DESCRIPTION
3100		NO ADVERSE FINDINGS
6827		NO ADVERSE FINDINGS
6828		NO ADVERSE FINDINGS
6829	DG(15- 20)	LOCALIZED ALOPECIA: LIMBS
6830		NO ADVERSE FINDINGS
6831		NO ADVERSE FINDINGS
6832	DG(6- 20)	LOCALIZED ALOPECIA: LIMBS a
	DG(9- 20)	LOCALIZED ALOPECIA: UNDERSIDE a
6833		NO ADVERSE FINDINGS
6834		NO ADVERSE FINDINGS
6835	DG(14)	RED SUBSTANCE IN CAGE PAN
	DG(14- 20)	PALE IN APPEARANCE
	DG(16)	RED SUBSTANCE IN CAGE PAN
	DG(16)	ABDOMINAL FUR: RED SUBSTANCE
	DG(16)	NO FECES
	DG(19- 20)	BROWN PERIVAGINAL SUBSTANCE a
	DG(20)	URINE-STAINED ABDOMINAL FUR a
	DG(20)	SACRIFICED; DAM IN THE PROCESS OF DELIVERY
6836		NO ADVERSE FINDINGS
6837		NO ADVERSE FINDINGS
6838		NO ADVERSE FINDINGS
6839		NO ADVERSE FINDINGS
6840		NO ADVERSE FINDINGS
6841		NO ADVERSE FINDINGS
6842		NO ADVERSE FINDINGS
6843		NO ADVERSE FINDINGS
6844		NO ADVERSE FINDINGS
6845		NO ADVERSE FINDINGS
6846		NO ADVERSE FINDINGS
6847		NO ADVERSE FINDINGS
6848		NO ADVERSE FINDINGS
6849		NO ADVERSE FINDINGS
6850		NO ADVERSE FINDINGS

DG = DAY OF PRESUMED GESTATION

a. Observation confirmed at necropsy.

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TABLE 13 (PAGE 3): CLINICAL OBSERVATIONS - INDIVIDUAL DATA

DOSAGE GROUP III		30 MG/KG/DAY
RAT #		DESCRIPTION
6851		NO ADVERSE FINDINGS
6852		NO ADVERSE FINDINGS
6853		NO ADVERSE FINDINGS
6854		NO ADVERSE FINDINGS
6855		NO ADVERSE FINDINGS
6856		NO ADVERSE FINDINGS
6857		NO ADVERSE FINDINGS
6858		NO ADVERSE FINDINGS
6859		NO ADVERSE FINDINGS
6860		NO ADVERSE FINDINGS
6861		NO ADVERSE FINDINGS
6862		NO ADVERSE FINDINGS
6863		NO ADVERSE FINDINGS
6864		NO ADVERSE FINDINGS
6865	DG(11)	SWOLLEN SNOUT
	DG(12)	SWELLING RESOLVED
6866		NO ADVERSE FINDINGS
6867		NO ADVERSE FINDINGS
6868		NO ADVERSE FINDINGS
6869		NO ADVERSE FINDINGS
6870		NO ADVERSE FINDINGS
6871		NO ADVERSE FINDINGS
6872		NO ADVERSE FINDINGS
6873		NO ADVERSE FINDINGS
6874		NO ADVERSE FINDINGS
6875		NO ADVERSE FINDINGS

DG = DAY OF PRESUMED GESTATION

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TABLE 13 (PAGE 4): CLINICAL OBSERVATIONS - INDIVIDUAL DATA

DOSAGE GROUP IV		100 MG/KG/DAY
RAT #		DESCRIPTION
6876		NO ADVERSE FINDINGS
6877		NO ADVERSE FINDINGS
6878		NO ADVERSE FINDINGS
6879		NO ADVERSE FINDINGS
6880		NO ADVERSE FINDINGS
6881	DG(10- 15)	LOCALIZED ALOPECIA: UNDERSIDE
	DG(16)	ALOPECIA NO LONGER APPARENT
6882		NO ADVERSE FINDINGS
6883		NO ADVERSE FINDINGS
6884		NO ADVERSE FINDINGS
6885		NO ADVERSE FINDINGS
6886		NO ADVERSE FINDINGS
6887		NO ADVERSE FINDINGS
6888		NO ADVERSE FINDINGS
6889		NO ADVERSE FINDINGS
6890	DG(11)	RALES
6891	DG(9)	RALES
6892		NO ADVERSE FINDINGS
6893		NO ADVERSE FINDINGS
6894		NO ADVERSE FINDINGS
6895		NO ADVERSE FINDINGS
6896		NO ADVERSE FINDINGS
6897		NO ADVERSE FINDINGS
6898		NO ADVERSE FINDINGS
6899		NO ADVERSE FINDINGS
6900		NO ADVERSE FINDINGS

DG = DAY OF PRESUMED GESTATION

PROTOCOL 720-002: DEVELOPMENTAL TOXICITY STUDY OF HYAMINE® 1622 IN RATS

TABLE 13 (PAGE 5): CLINICAL OBSERVATIONS - INDIVIDUAL DATA

DOSAGE GROUP V		170 MG/KG/DAY
RAT #		DESCRIPTION
6901	DG(8- 13)	SOFT FECES
	DG(10)	LIQUID FECES
	DG(10- 12)	URINE-STAINED ABDOMINAL FUR
	DG(12- 13)	EXCESS SALIVATION
	DG(12- 20)	LOCALIZED ALOPECIA: UNDERSIDE a
6902	DG(9- 12)	SOFT FECES
	DG(12- 20)	LOCALIZED ALOPECIA: BACK a
6903	DG(7- 11)	SOFT FECES
	DG(12)	EXCESS SALIVATION
	DG(14)	EXCESS SALIVATION
6904	DG(8- 16)	SOFT FECES
	DG(15)	NO FECES
	DG(16- 20)	LOCALIZED ALOPECIA: UNDERSIDE a
6905	DG(9- 11)	SOFT FECES
	DG(11- 20)	LOCALIZED ALOPECIA: UNDERSIDE a
6906	DG(14)	SOFT FECES
6907	DG(8)	SOFT FECES
6908	DG(5- 20)	LOCALIZED ALOPECIA: LIMBS a
	DG(8- 12)	SOFT FECES
	DG(9- 20)	LOCALIZED ALOPECIA: UNDERSIDE a
	DG(15)	SOFT FECES
6909	DG(12- 16)	SOFT FECES
6910	DG(7)	SOFT FECES
	DG(8)	EXCESS SALIVATION
	DG(8- 10)	LIQUID FECES
	DG(9- 11)	RALES
	DG(9- 11)	URINE-STAINED ABDOMINAL FUR a
	DG(9- 11)	RED PERINASAL SUBSTANCE a
	DG(9- 11)	FORELIMBS AND PAWS: RED SUBSTANCE
	DG(10- 11)	COLD TO TOUCH
	DG(10- 11)	NO FECES
	DG(11)	IMPAIRED RIGHTING REFLEX
	DG(11)	FOUND DEAD

DG = DAY OF PRESUMED GESTATION

a. Observation confirmed at necropsy.

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TABLE 13 (PAGE 6): CLINICAL OBSERVATIONS - INDIVIDUAL DATA

DOSAGE GROUP V		170 MG/KG/DAY
RAT #		DESCRIPTION
6911	DG(11- 17)	SOFT FECES
6912	DG(9)	SOFT FECES
	DG(11- 14)	SOFT FECES
6913	DG(8- 10)	SOFT FECES
	DG(10- 11)	LIQUID FECES
	DG(10- 14)	URINE-STAINED ABDOMINAL FUR
	DG(11- 13)	NO FECES
	DG(13- 15)	SOFT FECES
	DG(17)	SOFT FECES
6914	DG(9- 10)	SOFT FECES
	DG(12- 17)	SOFT FECES
	DG(14- 20)	LOCALIZED ALOPECIA: LIMBS a
	DG(17- 20)	LOCALIZED ALOPECIA: UNDERSIDE a
6915	DG(9- 13)	SOFT FECES
6916		NO ADVERSE FINDINGS
6917	DG(7- 20)	LOCALIZED ALOPECIA: UNDERSIDE a
	DG(8- 15)	SOFT FECES
	DG(14)	VOCALIZATION
	DG(14- 15)	EXCESS SALIVATION
	DG(14- 15)	DYSPNEA
	DG(15- 16)	FECES-STAINED FUR
	DG(15- 16)	LIQUID FECES
	DG(17)	SOFT FECES
6918	DG(8)	SOFT FECES
	DG(9- 12)	URINE-STAINED ABDOMINAL FUR a
	DG(9- 12)	RED PERINASAL SUBSTANCE a
	DG(9- 12)	LIQUID FECES
	DG(10)	NO FECES
	DG(11- 12)	FORELIMBS AND PAWS: RED SUBSTANCE a
	DG(12)	PTOSIS
	DG(12)	COLD TO TOUCH
	DG(12)	FECES-STAINED FUR a
	DG(13)	FOUND DEAD

DG = DAY OF PRESUMED GESTATION

a. Observation confirmed at necropsy.

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TABLE 13 (PAGE 7): CLINICAL OBSERVATIONS - INDIVIDUAL DATA

DOSAGE GROUP V		170 MG/KG/DAY
RAT #		DESCRIPTION
6919	DG(9- 10)	SOFT FECES
	DG(12- 14)	SOFT FECES
6920	DG(10)	SOFT FECES
	DG(14)	NO FECES
6921	DG(8)	FECES-STAINED FUR
	DG(8)	SOFT FECES
	DG(8)	NO FECES
	DG(8- 10)	LIQUID FECES
	DG(9- 10)	URINE-STAINED ABDOMINAL FUR
	DG(10- 15)	SOFT FECES
	DG(13)	EXCESS SALIVATION
	DG(14- 16)	DYSPNEA
	DG(15- 16)	PALE IN APPEARANCE
	DG(15- 16)	URINE-STAINED ABDOMINAL FUR ^a
	DG(15- 16)	NO FECES
	DG(16)	COLD TO TOUCH
	DG(16)	RED PERINASAL SUBSTANCE ^a
	DG(16)	FORELIMBS AND PAWS: RED SUBSTANCE ^a
	DG(16)	FOUND DEAD
6922	DG(9)	SOFT FECES
	DG(11- 14)	SOFT FECES
	DG(16)	SOFT FECES
6923	DG(7- 11)	SOFT FECES
	DG(10- 11)	EXCESS SALIVATION
	DG(12)	FOUND DEAD
6924	DG(11)	NO FECES
	DG(12)	SOFT FECES
	DG(15)	SOFT FECES
6925	DG(9- 16)	SOFT FECES

DG = DAY OF PRESUMED GESTATION

^a. Observation confirmed at necropsy.

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TABLE 14 (PAGE 1): MATERNAL BODY WEIGHTS AND UTERINE WEIGHT - INDIVIDUAL DATA

PREGNANCY STATUS	DAY	0	6	9	12	15	16	18	20	UTERINE WEIGHT
RAT #	DOSAGE GROUP	I	0(VEHICLE) MG/KG/DAY							
6801 P	251.	289.	295.	316.	333.	339.	369.	401.		72.46
6802 P	241.	272.	275.	293.	302.	312.	348.	370.		73.80
6803 P	222.	260.	263.	288.	305.	312.	342.	369.		66.64
6804 P	237.	269.	276.	293.	312.	317.	356.	394.		85.45
6805 P	221.	251.	258.	281.	292.	308.	346.	377.		78.01
6806 P	233.	277.	292.	308.	326.	335.	370.	398.		84.31
6807 P	227.	262.	265.	287.	299.	306.	337.	371.		75.72
6808 P	258.	295.	309.	340.	356.	368.	394.	436.		78.86
6809 P	227.	253.	258.	272.	294.	295.	322.	350.		58.73
6810 P	233.	262.	269.	292.	308.	321.	353.	388.		87.71
6811 P	239.	265.	267.	279.	297.	307.	338.	360.		72.88
6812 P	228.	253.	258.	271.	290.	300.	332.	357.		68.60
6813 P	246.	274.	280.	297.	327.	345.	378.	408.		91.46
6814 P	230.	257.	260.	276.	297.	308.	344.	372.		78.43
6815 P	231.	266.	278.	300.	317.	335.	364.	400.		97.25
6816 P	230.	239.	260.	268.	283.	298.	323.	350.		75.72
6817 P	234.	269.	284.	295.	310.	318.	351.	375.		79.27
6818 P	237.	263.	270.	284.	307.	314.	351.	381.		73.63
6819 P	224.	262.	271.	284.	299.	310.	336.	353.		64.08
6820 P	226.	259.	281.	294.	324.	330.	366.	403.		83.07
6821 P	218.	252.	270.	286.	320.	323.	356.	396.		100.63
6822 P	239.	279.	295.	315.	339.	351.	377.	413.		91.74
6823 P	251.	277.	287.	307.	324.	334.	357.	394.		84.75
6824 P	244.	284.	291.	314.	336.	350.	376.	405.		73.43
6825 P	236.	261.	280.	292.	325.	331.	363.	394.		91.67

P = PREGNANT NP = NOT PREGNANT (VALUES EXCLUDED FROM AVERAGES)

DAY = DAY OF PRESUMED GESTATION

ALL WEIGHTS WERE RECORDED IN GRAMS (G).

PROTOCOL 720-002: DEVELOPMENTAL TOXICITY STUDY OF HYAMINE® 1622 IN RATS

TABLE 14 (PAGE 2): MATERNAL BODY WEIGHTS AND UTERINE WEIGHT - INDIVIDUAL DATA

PREGNANCY STATUS		DAY 0	6	9	12	15	16	18	20	UTERINE WEIGHT
RAT #	DOSAGE GROUP II	10 MG/KG/DAY								
3100 P		217.	239.	256.	271.	289.	299.	331.	363.	92.16
6827 P		228.	258.	264.	284.	300.	305.	334.	367.	70.08
6828 P		250.	272.	277.	291.	306.	315.	355.	385.	82.66
6829 P		223.	268.	279.	301.	316.	318.	353.	381.	66.08
6830 P		232.	270.	282.	307.	327.	336.	376.	414.	83.91
6831 P		236.	274.	283.	311.	329.	340.	378.	411.	92.52
6832 P		236.	258.	260.	281.	298.	310.	347.	383.	86.49
6833 P		241.	266.	268.	287.	308.	311.	338.	371.	71.37
6834 P		239.	262.	274.	296.	304.	312.	349.	374.	77.20
6835 P		247.	268.	274.	270.	246.	249.	261.	SACRIFICED ON DAY 20 OF GESTATION a	
6836 P		224.	266.	281.	295.	312.	326.	349.	387.	80.95
6837 P		239.	258.	268.	287.	304.	311.	346.	371.	71.74
6838 P		233.	255.	267.	286.	301.	314.	338.	366.	74.23
6839 P		230.	265.	283.	297.	318.	336.	371.	409.	96.68
6840 P		227.	258.	274.	287.	308.	310.	340.	361.	59.97
6841 P		232.	261.	272.	285.	307.	315.	343.	369.	65.59
6842 P		226.	250.	245.	270.	289.	301.	335.	363.	76.83
6843 P		221.	252.	257.	276.	289.	306.	330.	356.	56.78
6844 P		229.	244.	257.	275.	290.	304.	330.	360.	69.00
6845 P		243.	275.	293.	306.	330.	345.	372.	392.	75.88
6846 P		237.	261.	272.	297.	310.	329.	374.	405.	91.55
6847 NP		234.	250.	248.	240.	249.	247.	251.	257.	0.43
6848 P		218.	236.	252.	268.	290.	297.	332.	359.	79.44
6849 P		252.	278.	290.	308.	331.	344.	376.	403.	80.51
6850 P		258.	284.	293.	305.	327.	339.	358.	390.	78.56

P = PREGNANT NP = NOT PREGNANT (VALUES EXCLUDED FROM AVERAGES)

DAY = DAY OF PRESUMED GESTATION

ALL WEIGHTS WERE RECORDED IN GRAMS (G).

a. The dam was in the process of delivery.

PROTOCOL 720-002: DEVELOPMENTAL TOXICITY STUDY OF HYAMINE® 1622 IN RATS

TABLE 14 (PAGE 3): MATERNAL BODY WEIGHTS AND UTERINE WEIGHT - INDIVIDUAL DATA

PREGNANCY		DAY	0	6	9	12	15	16	18	20	UTERINE
STATUS											
RAT #		DOSAGE GROUP III									
		30 MG/KG/DAY									
6851	NP	242.	246.	239.	251.	234.	243.	254.	245.		0.53
6852	P	236.	271.	263.	275.	307.	313.	348.	378.		84.72
6853	P	231.	264.	272.	295.	304.	315.	357.	392.		86.42
6854	P	223.	257.	256.	276.	293.	300.	340.	369.		70.15
6855	P	237.	268.	275.	293.	307.	315.	344.	378.		68.48
6856	P	240.	255.	274.	290.	302.	312.	338.	373.		85.17
6857	P	234.	268.	272.	295.	310.	310.	337.	366.		57.96
6858	P	253.	290.	299.	325.	336.	349.	381.	410.		81.07
6859	P	228.	267.	269.	296.	310.	315.	347.	385.		73.79
6860	P	239.	279.	298.	318.	339.	344.	386.	418.		a
6861	P	220.	240.	246.	268.	277.	289.	315.	346.		67.45
6862	P	232.	257.	269.	283.	309.	317.	346.	370.		77.07
6863	P	224.	252.	264.	278.	303.	312.	341.	371.		77.73
6864	P	231.	255.	246.	266.	290.	306.	337.	371.		81.46
6865	P	240.	263.	273.	290.	305.	317.	357.	391.		86.70
6866	P	236.	262.	277.	296.	317.	328.	357.	380.		78.50
6867	P	241.	273.	283.	305.	324.	335.	362.	394.		70.85
6868	P	227.	254.	261.	282.	291.	306.	330.	357.		69.31
6869	P	230.	249.	253.	266.	283.	295.	323.	346.		74.60
6870	P	233.	249.	265.	277.	300.	303.	332.	353.		56.06
6871	P	257.	286.	304.	320.	343.	349.	378.	410.		83.32
6872	P	224.	240.	239.	249.	261.	271.	293.	323.		67.98
6873	P	218.	238.	242.	254.	275.	283.	313.	335.		78.44
6874	P	245.	275.	284.	302.	317.	322.	349.	376.		79.40
6875	P	247.	279.	302.	316.	339.	355.	382.	410.		80.10

P = PREGNANT NP = NOT PREGNANT (VALUES EXCLUDED FROM AVERAGES)

DAY = DAY OF PRESUMED GESTATION

ALL WEIGHTS WERE RECORDED IN GRAMS (G).

a. Value was not recorded.

PROTOCOL 720-002: DEVELOPMENTAL TOXICITY STUDY OF HYAMINE® 1622 IN RATS

TABLE 14 (PAGE 4): MATERNAL BODY WEIGHTS AND UTERINE WEIGHT - INDIVIDUAL DATA

PREGNANCY STATUS		DAY 0	6	9	12	15	16	18	20	UTERINE WEIGHT
RAT #		DOSAGE GROUP IV 100 MG/KG/DAY								
6876 P		229.	255.	269.	287.	298.	307.	340.	369.	79.43
6877 P		245.	283.	289.	309.	321.	325.	359.	389.	68.19
6878 P		231.	265.	270.	291.	300.	310.	344.	375.	82.13
6879 P		236.	296.	301.	327.	348.	353.	391.	418.	66.08
6880 P		225.	262.	272.	289.	304.	314.	347.	380.	75.76
6881 NP		243.	273.	285.	280.	276.	278.	270.	273.	0.53
6882 P		234.	265.	273.	303.	306.	319.	355.	382.	58.61
6883 P		220.	253.	258.	276.	288.	299.	327.	360.	77.13
6884 P		228.	260.	273.	293.	305.	317.	349.	379.	77.24
6885 P		252.	289.	305.	323.	335.	348.	368.	392.	47.99
6886 P		238.	272.	276.	297.	310.	323.	351.	378.	73.16
6887 P		240.	266.	276.	295.	310.	318.	347.	372.	66.64
6888 P		233.	252.	261.	277.	304.	309.	339.	370.	79.26
6889 P		247.	277.	288.	303.	316.	322.	353.	391.	82.01
6890 P		222.	262.	272.	293.	311.	321.	352.	377.	73.03
6891 P		230.	250.	236.	259.	281.	288.	319.	338.	73.67
6892 P		233.	261.	271.	283.	307.	315.	347.	381.	86.17
6893 P		224.	251.	255.	275.	290.	293.	332.	356.	67.68
6894 P		239.	263.	275.	286.	300.	304.	332.	363.	76.57
6895 P		236.	253.	265.	282.	298.	308.	333.	362.	79.02
6896 P		240.	278.	285.	297.	316.	320.	356.	390.	72.49
6897 P		220.	246.	257.	269.	287.	300.	326.	350.	69.32
6898 P		259.	292.	302.	324.	348.	361.	392.	423.	86.88
6899 P		237.	270.	278.	271.	298.	301.	336.	368.	74.54
6900 P		226.	281.	291.	317.	342.	350.	383.	404.	76.28

P = PREGNANT NP = NOT PREGNANT (VALUES EXCLUDED FROM AVERAGES)

DAY = DAY OF PRESUMED GESTATION

ALL WEIGHTS WERE RECORDED IN GRAMS (G).

PROTOCOL 720-002: DEVELOPMENTAL TOXICITY STUDY OF HYAMINE® 1622 IN RATS

TABLE 14 (PAGE 5): MATERNAL BODY WEIGHTS AND UTERINE WEIGHT - INDIVIDUAL DATA

PREGNANCY		DAY 0	6	9	12	15	16	18	20	UTERINE
STATUS										WEIGHT
RAT #	DOSAGE GROUP V	170 MG/KG/DAY								
6901 P		218.	256.	235.	230.	268.	280.	299.	336.	59.16
6902 P		240.	265.	274.	282.	318.	323.	356.	386.	81.57
6903 P		223.	255.	255.	286.	308.	320.	348.	388.	100.51
6904 P		239.	264.	256.	266.	256.	265.	321.	363.	77.68
6905 P		234.	265.	271.	299.	313.	327.	360.	390.	94.48
6906 P		223.	261.	269.	278.	295.	296.	331.	370.	79.49
6907 P		226.	250.	247.	271.	280.	282.	288.	317.	30.54
6908 P		221.	252.	257.	275.	287.	294.	320.	357.	60.92
6909 P		236.	269.	275.	287.	294.	303.	332.	356.	63.87
6910 P		256.	269.	213.	FOUND DEAD ON DAY 11 OF GESTATION					-----
6911 P		232.	254.	274.	268.	282.	285.	317.	357.	71.28
6912 P		240.	261.	272.	280.	290.	303.	319.	339.	54.56
6913 P		227.	248.	238.	217.	246.	259.	285.	322.	62.22
6914 P		231.	258.	268.	271.	286.	294.	326.	357.	76.30
6915 P		231.	251.	254.	261.	291.	301.	328.	359.	72.62
6916 P		239.	266.	278.	293.	313.	328.	354.	378.	75.18
6917 P		243.	280.	284.	301.	258.	269.	296.	309.	34.75
6918 P		236.	269.	231.	186.	FOUND DEAD ON DAY 13 OF GESTATION				-----
6919 P		237.	264.	266.	279.	293.	301.	320.	333.	32.14
6920 P		234.	261.	274.	289.	305.	317.	340.	369.	72.78
6921 P		252.	270.	247.	252.	218.	210.	FOUND DEAD ON DAY 16 OF GESTATION		-----
6922 P		245.	269.	276.	276.	284.	290.	320.	346.	67.05
6923 P		250.	270.	255.	FOUND DEAD ON DAY 12 OF GESTATION				-----	-----
6924 P		229.	260.	271.	283.	301.	312.	337.	357.	67.43
6925 P		224.	260.	257.	271.	301.	315.	340.	381.	93.35

P = PREGNANT NP = NOT PREGNANT (VALUES EXCLUDED FROM AVERAGES)

DAY = DAY OF PRESUMED GESTATION

ALL WEIGHTS WERE RECORDED IN GRAMS (G).

PROTOCOL 720-002: DEVELOPMENTAL TOXICITY STUDY OF HYAMINE® 1622 IN RATS

TABLE 15 (PAGE 1): MATERNAL FEED CONSUMPTION VALUES - INDIVIDUAL DATA

PREGNANCY						
STATUS	DAYS	0 - 6	6 - 9	9 - 12	12 - 16	16 - 18 18 - 20
RAT #	DOSAGE GROUP I	0(VEHICLE) MG/KG/DAY				
6801 P	143.	76.	81.	108.	59.	55.
6802 P	135.	66.	73.	96.	59.	49.
6803 P	127.	67.	78.	104.	52.	54.
6804 P	128.	65.	69.	97.	56.	55.
6805 P	113.	63.	78.	101.	66.	63.
6806 P	133.	70.	75.	102.	59.	54.
6807 P	132.	67.	73.	98.	56.	55.
6808 P	143.	77.	84.	110.	57.	60.
6809 P	119.	46.	57.	92.	50.	49.
6810 P	129.	62.	70.	98.	54.	55.
6811 P	121.	60.	66.	93.	51.	48.
6812 P	119.	60.	67.	96.	55.	50.
6813 P	133.	70.	69.	103.	61.	58.
6814 P	129.	63.	65.	94.	57.	52.
6815 P	120.	71.	74.	106.	58.	55.
6816 P	94.	66.	64.	86.	51.	50.
6817 P	143.	74.	75.	99.	58.	52.
6818 P	113.	61.	73.	101.	58.	57.
6819 P	147.	77.	80.	114.	55.	51.
6820 P	120.	79.	77.	121.	62.	61.
6821 P	136.	63.	75.	102.	56.	57.
6822 P	149.	84.	83.	119.	56.	58.
6823 P	144.	77.	80.	106.	51.	57.
6824 P	142.	74.	81.	112.	58.	58.
6825 P	121.	68.	72.	102.	53.	53.

P = PREGNANT NP = NOT PREGNANT (VALUES EXCLUDED FROM AVERAGES)
DAYS = DAYS OF PRESUMED GESTATION
ALL WEIGHTS WERE RECORDED IN GRAMS (G).

PROTOCOL 720-002: DEVELOPMENTAL TOXICITY STUDY OF HYAMINE® 1622 IN RATS

TABLE 15 (PAGE 2): MATERNAL FEED CONSUMPTION VALUES - INDIVIDUAL DATA

PREGNANCY						
STATUS	DAYS	0 - 6	6 - 9	9 - 12	12 - 16	16 - 18 18 - 20
RAT #	DOSAGE GROUP II					
	10 MG/KG/DAY					
3100 P	121.	65.	69.	90.	50.	42.
6827 P	121.	62.	70.	94.	51.	54.
6828 P	123.	62.	53.	98.	56.	55.
6829 P	132.	72.	75.	104.	63.	59.
6830 P	130.	71.	77.	106.	63.	60.
6831 P	145.	75.	76.	99.	61.	60.
6832 P	110.	61.	67.	95.	55.	55.
6833 P	116.	59.	64.	91.	48.	53.
6834 P	125.	69.	70.	91.	54.	54.
6835 P	98.	54.	38.	26.	22.	SACRIFICED ON DAY 20 OF GESTATION a
6836 P	133.	72.	76.	102.	54.	53.
6837 P	135.	72.	76.	107.	61.	51.
6838 P	122.	68.	69.	97.	53.	52.
6839 P	164.	88.	89.	122.	64.	58.
6840 P	138.	74.	76.	108.	54.	51.
6841 P	127.	64.	67.	93.	50.	50.
6842 P	115.	38.	62.	90.	53.	46.
6843 P	124.	62.	65.	94.	56.	52.
6844 P	91.	66.	66.	95.	51.	49.
6845 P	111.	73.	76.	114.	61.	50.
6846 P	128.	62.	79.	95.	62.	56.
6847 NP	111.	53.	49.	67.	33.	39.
6848 P	111.	66.	69.	96.	54.	46.
6849 P	128.	68.	76.	108.	60.	55.
6850 P	142.	70.	74.	103.	51.	55.

P = PREGNANT NP = NOT PREGNANT (VALUES EXCLUDED FROM AVERAGES)

DAYS = DAYS OF PRESUMED GESTATION

ALL WEIGHTS WERE RECORDED IN GRAMS (G).

a. The dam was in the process of delivery.

PROTOCOL 720-002: DEVELOPMENTAL TOXICITY STUDY OF HYAMINE® 1622 IN RATS

TABLE 15 (PAGE 3): MATERNAL FEED CONSUMPTION VALUES - INDIVIDUAL DATA

PREGNANCY						
STATUS	DAYS	0 - 6	6 - 9	9 - 12	12 - 16	16 - 18 18 - 20
RAT #	DOSAGE GROUP III			30 MG/KG/DAY		
6851 NP	108.	49.	58.	54.	43.	32.
6852 P	117.	43.	58.	99.	56.	51.
6853 P	128.	64.	74.	93.	59.	55.
6854 P	117.	56.	59.	87.	54.	49.
6855 P	128.	66.	69.	92.	52.	51.
6856 P	111.	70.	73.	92.	53.	52.
6857 P	124.	64.	70.	95.	54.	53.
6858 P	153.	78.	78.	98.	56.	53.
6859 P	128.	62.	72.	89.	56.	56.
6860 P	134.	75.	78.	108.	65.	58.
6861 P	111.	57.	60.	84.	48.	49.
6862 P	126.	63.	67.	98.	55.	49.
6863 P	123.	63.	68.	91.	50.	47.
6864 P	136.	39.	66.	104.	56.	53.
6865 P	130.	66.	70.	98.	60.	55.
6866 P	131.	70.	73.	103.	55.	50.
6867 P	132.	69.	73.	106.	60.	59.
6868 P	116.	60.	65.	91.	49.	48.
6869 P	116.	51.	61.	92.	51.	44.
6870 P	97.	57.	63.	94.	52.	50.
6871 P	143.	73.	73.	98.	51.	52.
6872 P	119.	49.	56.	77.	44.	46.
6873 P	110.	52.	55.	82.	44.	39.
6874 P	142.	69.	71.	131. ^a	47.	49.
6875 P	151.	81.	89.	123.	67.	61.

P = PREGNANT NP = NOT PREGNANT (VALUES EXCLUDED FROM AVERAGES)

DAYS = DAYS OF PRESUMED GESTATION

ALL WEIGHTS WERE RECORDED IN GRAMS (G).

a. Value was associated with spillage and was excluded from group averages and statistical analyses.

PROTOCOL 720-002: DEVELOPMENTAL TOXICITY STUDY OF HYAMINE® 1622 IN RATS

TABLE 15 (PAGE 4): MATERNAL FEED CONSUMPTION VALUES - INDIVIDUAL DATA

PREGNANCY						
STATUS	DAYS	0 - 6	6 - 9	9 - 12	12 - 16	16 - 18 18 - 20
RAT #	DOSAGE GROUP IV	100 MG/KG/DAY				
6876 P	127.	71.	76.	92.	56.	57.
6877 P	141.	69.	75.	93.	54.	53.
6878 P	132.	62.	72.	92.	59.	55.
6879 P	179.	85.	97.	123.	76.	73.
6880 P	128.	59.	68.	87.	58.	56.
6881 NP	125.	61.	57.	70.	30.	35.
6882 P	133.	62.	69.	90.	60.	57.
6883 P	131.	63.	65.	81.	51.	54.
6884 P	133.	64.	70.	89.	57.	54.
6885 P	149.	66.	81.	100.	55.	61.
6886 P	135.	62.	69.	97.	56.	52.
6887 P	129.	65.	71.	96.	60.	53.
6888 P	133.	57.	65.	95.	56.	54.
6889 P	139.	65.	67.	88.	53.	52.
6890 P	137.	73.	78.	105.	59.	55.
6891 P	129.	51.	58.	88.	53.	47.
6892 P	135.	63.	68.	99.	56.	52.
6893 P	122.	49.	58.	90.	60.	51.
6894 P	133.	64.	69.	85.	51.	48.
6895 P	110.	54.	60.	83.	49.	44.
6896 P	136.	64.	69.	94.	58.	55.
6897 P	129.	61.	65.	93.	50.	46.
6898 P	145.	69.	80.	107.	61.	60.
6899 P	137.	60.	51.	85.	58.	56.
6900 P	172.	85.	97.	119.	63.	55.

P = PREGNANT NP = NOT PREGNANT (VALUES EXCLUDED FROM AVERAGES)

DAYS = DAYS OF PRESUMED GESTATION

ALL WEIGHTS WERE RECORDED IN GRAMS (G).

PROTOCOL 720-002: DEVELOPMENTAL TOXICITY STUDY OF HYAMINE® 1622 IN RATS

TABLE 15 (PAGE 5): MATERNAL FEED CONSUMPTION VALUES - INDIVIDUAL DATA

PREGNANCY						
STATUS	DAYS	0 - 6	6 - 9	9 - 12	12 - 16	16 - 18 18 - 20
RAT #	DOSAGE	GROUP V 170 MG/KG/DAY				
6901 P	128.	30.	20.	100.	61.	65.
6902 P	120.	49.	52.	101.	65.	56.
6903 P	122.	39.	68.	102.	62.	55.
6904 P	120.	36.	49.	56.	59.	59.
6905 P	136.	54.	65.	103.	66.	60.
6906 P	140.	60.	67.	95.	64.	61.
6907 P	116.	35.	58.	89.	52.	61.
6908 P	113.	36.	58.	80.	58.	64.
6909 P	137.	58.	59.	65.	61.	55.
6910 P	126.	a	FOUND DEAD ON DAY 11 OF GESTATION			
6911 P	134.	64.	44.	65.	60.	63.
6912 P	130.	50.	55.	82.	56.	49.
6913 P	113.	32.	14.	71.	57.	55.
6914 P	124.	52.	56.	83.	57.	54.
6915 P	120.	47.	48.	91.	60.	59.
6916 P	126.	58.	74.	104.	64.	53.
6917 P	146.	51.	57.	37.	53.	53.
6918 P	134.	16.	0.	FOUND DEAD ON DAY 13 OF GESTATION		
6919 P	133.	41.	56.	86.	57.	49.
6920 P	135.	60.	57.	95.	57.	56.
6921 P	119.	24.	31.	33.	FOUND DEAD ON DAY 16 OF GESTATION	
6922 P	140.	58.	47.	55.	52.	55.
6923 P	125.	32.	FOUND DEAD ON DAY 12 OF GESTATION			
6924 P	133.	56.	63.	89.	50.	48.
6925 P	146.	47.	58.	98.	63.	64.

P = PREGNANT NP = NOT PREGNANT (VALUES EXCLUDED FROM AVERAGES)

DAYS = DAYS OF PRESUMED GESTATION

ALL WEIGHTS WERE RECORDED IN GRAMS (G).

a. Soiled feed precluded the calculation of this value.

PROTOCOL 720-002: DEVELOPMENTAL TOXICITY STUDY OF HYAMINE® 1622 IN RATS

TABLE 16 (PAGE 1): NECROPSY OBSERVATIONS - INDIVIDUAL DATA

DOSAGE GROUP DOSAGE (MG/KG/DAY)	RAT NUMBER	DAY OF NECROPSY	PREGNANCY STATUS	DOSAGES ADMINISTERED	OBSERVATIONS
I 0 (VEHICLE)	6801	DG 20	P	10	ALL TISSUES APPEARED NORMAL.
	6802	DG 20	P	10	ALL TISSUES APPEARED NORMAL.
	6803	DG 20	P	10	ALL TISSUES APPEARED NORMAL.
	6804	DG 20	P	10	ALL TISSUES APPEARED NORMAL.
	6805	DG 20	P	10	ALL TISSUES APPEARED NORMAL.
	6806	DG 20	P	10	ALL TISSUES APPEARED NORMAL.
	6807	DG 20	P	10	ALL TISSUES APPEARED NORMAL.
	6808	DG 20	P	10	ALL TISSUES APPEARED NORMAL.
	6809	DG 20	P	10	ALL TISSUES APPEARED NORMAL.
	6810	DG 20	P	10	ALL TISSUES APPEARED NORMAL.
	6811	DG 20	P	10	ALL TISSUES APPEARED NORMAL.
	6812	DG 20	P	10	ALL TISSUES APPEARED NORMAL.
	6813	DG 20	P	10	ALL TISSUES APPEARED NORMAL.
	6814	DG 20	P	10	ALL TISSUES APPEARED NORMAL.
	6815	DG 20	P	10	ALL TISSUES APPEARED NORMAL.
	6816	DG 20	P	10	ALL TISSUES APPEARED NORMAL.
	6817	DG 20	P	10	ALL TISSUES APPEARED NORMAL.
	6818	DG 20	P	10	ALL TISSUES APPEARED NORMAL.
	6819	DG 20	P	10	ALL TISSUES APPEARED NORMAL.
	6820	DG 20	P	10	ALL TISSUES APPEARED NORMAL.
	6821	DG 20	P	10	ALL TISSUES APPEARED NORMAL.
	6822	DG 20	P	10	ALL TISSUES APPEARED NORMAL.
	6823	DG 20	P	10	ALL TISSUES APPEARED NORMAL.
	6824	DG 20	P	10	ALL TISSUES APPEARED NORMAL.
	6825	DG 20	P	10	ALL TISSUES APPEARED NORMAL.

P = PREGNANT NP = NOT PREGNANT
DG = DAY OF PRESUMED GESTATION

PROTOCOL 720-002: DEVELOPMENTAL TOXICITY STUDY OF HYAMINE® 1622 IN RATS

TABLE 16 (PAGE 2): NECROPSY OBSERVATIONS - INDIVIDUAL DATA

DOSAGE GROUP DOSAGE (MG/KG/DAY)	RAT NUMBER	DAY OF NECROPSY	PREGNANCY STATUS	DOSAGES ADMINISTERED	OBSERVATIONS
II 10	3100	DG 20	P	10	ALL TISSUES APPEARED NORMAL.
	6827	DG 20	P	10	ALL TISSUES APPEARED NORMAL.
	6828	DG 20	P	10	ALL TISSUES APPEARED NORMAL.
	6829	DG 20	P	10	ALL TISSUES APPEARED NORMAL.
	6830	DG 20	P	10	ALL TISSUES APPEARED NORMAL.
	6831	DG 20	P	10	ALL TISSUES APPEARED NORMAL.
	6832	DG 20	P	10	ALL TISSUES APPEARED NORMAL.
	6833	DG 20	P	10	ALL TISSUES APPEARED NORMAL.
	6834	DG 20	P	10	ALL TISSUES APPEARED NORMAL.
	6835	DG 20	P	10	SACRIFICED ON DAY 20 OF GESTATION; DAM IN THE PROCESS OF DELIVERY. THYMUS: SMALL (0.051 G) AND DARK RED. STOMACH: RED SUBSTANCE. LIVER: PALE. SPLEEN: LARGE (8.0 CM X 2.0 CM X 1.0 CM, 82.06 G). ALL OTHER TISSUES APPEARED NORMAL. UTERINE CONTENTS: 15 IMPLANTATION SITES (ONE EARLY RESORPTION AND NINE LATE RESORPTIONS). ^a
	6836	DG 20	P	10	ALL TISSUES APPEARED NORMAL.
	6837	DG 20	P	10	ALL TISSUES APPEARED NORMAL.
	6838	DG 20	P	10	ALL TISSUES APPEARED NORMAL.
	6839	DG 20	P	10	ALL TISSUES APPEARED NORMAL.
	6840	DG 20	P	10	ALL TISSUES APPEARED NORMAL.
	6841	DG 20	P	10	ALL TISSUES APPEARED NORMAL.
	6842	DG 20	P	10	ALL TISSUES APPEARED NORMAL.
	6843	DG 20	P	10	ALL TISSUES APPEARED NORMAL.
	6844	DG 20	P	10	ALL TISSUES APPEARED NORMAL.
	6845	DG 20	P	10	ALL TISSUES APPEARED NORMAL.
	6846	DG 20	P	10	ALL TISSUES APPEARED NORMAL.
	6847	DG 20	NP	10	ALL TISSUES APPEARED NORMAL.
	6848	DG 20	P	10	ALL TISSUES APPEARED NORMAL.
	6849	DG 20	P	10	ALL TISSUES APPEARED NORMAL.
	6850	DG 20	P	10	ALL TISSUES APPEARED NORMAL.

P = PREGNANT NP = NOT PREGNANT

DG = DAY OF PRESUMED GESTATION

a. The remaining conceptuses were presumed to have been cannibalized.

PROTOCOL 720-002: DEVELOPMENTAL TOXICITY STUDY OF HYAMINE® 1622 IN RATS

TABLE 16 (PAGE 3): NECROPSY OBSERVATIONS - INDIVIDUAL DATA

DOSAGE GROUP DOSAGE (MG/KG/DAY)	RAT NUMBER	DAY OF NECROPSY	PREGNANCY STATUS	DOSAGES ADMINISTERED	OBSERVATIONS
III 30	6851	DG 20	NP	10	ALL TISSUES APPEARED NORMAL.
	6852	DG 20	P	10	ALL TISSUES APPEARED NORMAL.
	6853	DG 20	P	10	ALL TISSUES APPEARED NORMAL.
	6854	DG 20	P	10	ALL TISSUES APPEARED NORMAL.
	6855	DG 20	P	10	ALL TISSUES APPEARED NORMAL.
	6856	DG 20	P	10	ALL TISSUES APPEARED NORMAL.
	6857	DG 20	P	10	ALL TISSUES APPEARED NORMAL.
	6858	DG 20	P	10	ALL TISSUES APPEARED NORMAL.
	6859	DG 20	P	10	ALL TISSUES APPEARED NORMAL.
	6860	DG 20	P	10	ALL TISSUES APPEARED NORMAL.
	6861	DG 20	P	10	ALL TISSUES APPEARED NORMAL.
	6862	DG 20	P	10	ALL TISSUES APPEARED NORMAL.
	6863	DG 20	P	10	ALL TISSUES APPEARED NORMAL.
	6864	DG 20	P	10	ALL TISSUES APPEARED NORMAL.
	6865	DG 20	P	10	ALL TISSUES APPEARED NORMAL.
	6866	DG 20	P	10	ALL TISSUES APPEARED NORMAL.
	6867	DG 20	P	10	ALL TISSUES APPEARED NORMAL.
	6868	DG 20	P	10	ALL TISSUES APPEARED NORMAL.
	6869	DG 20	P	10	ALL TISSUES APPEARED NORMAL.
	6870	DG 20	P	10	ALL TISSUES APPEARED NORMAL.
	6871	DG 20	P	10	ALL TISSUES APPEARED NORMAL.
	6872	DG 20	P	10	ALL TISSUES APPEARED NORMAL.
	6873	DG 20	P	10	ALL TISSUES APPEARED NORMAL.
	6874	DG 20	P	10	ALL TISSUES APPEARED NORMAL.
	6875	DG 20	P	10	ALL TISSUES APPEARED NORMAL.

P = PREGNANT NP = NOT PREGNANT

DG = DAY OF PRESUMED GESTATION

PROTOCOL 720-002: DEVELOPMENTAL TOXICITY STUDY OF HYAMINE® 1622 IN RATS

TABLE 16 (PAGE 4): NECROPSY OBSERVATIONS - INDIVIDUAL DATA

DOSAGE GROUP DOSAGE (MG/KG/DAY)	RAT NUMBER	DAY OF NECROPSY	PREGNANCY STATUS	DOSAGES ADMINISTERED	OBSERVATIONS
IV 100	6876	DG 20	P	10	ALL TISSUES APPEARED NORMAL.
	6877	DG 20	P	10	ALL TISSUES APPEARED NORMAL.
	6878	DG 20	P	10	ALL TISSUES APPEARED NORMAL.
	6879	DG 20	P	10	ALL TISSUES APPEARED NORMAL.
	6880	DG 20	P	10	ALL TISSUES APPEARED NORMAL.
	6881	DG 20	NP	10	ALL TISSUES APPEARED NORMAL.
	6882	DG 20	P	10	ALL TISSUES APPEARED NORMAL.
	6883	DG 20	P	10	ALL TISSUES APPEARED NORMAL.
	6884	DG 20	P	10	ALL TISSUES APPEARED NORMAL.
	6885	DG 20	P	10	ALL TISSUES APPEARED NORMAL.
	6886	DG 20	P	10	ALL TISSUES APPEARED NORMAL.
	6887	DG 20	P	10	ALL TISSUES APPEARED NORMAL.
	6888	DG 20	P	10	ALL TISSUES APPEARED NORMAL.
	6889	DG 20	P	10	ALL TISSUES APPEARED NORMAL.
	6890	DG 20	P	10	ALL TISSUES APPEARED NORMAL.
	6891	DG 20	P	10	ALL TISSUES APPEARED NORMAL.
	6892	DG 20	P	10	ALL TISSUES APPEARED NORMAL.
	6893	DG 20	P	10	ALL TISSUES APPEARED NORMAL.
	6894	DG 20	P	10	ALL TISSUES APPEARED NORMAL.
	6895	DG 20	P	10	ALL TISSUES APPEARED NORMAL.
	6896	DG 20	P	10	ALL TISSUES APPEARED NORMAL.
	6897	DG 20	P	10	ALL TISSUES APPEARED NORMAL.
	6898	DG 20	P	10	ALL TISSUES APPEARED NORMAL.
	6899	DG 20	P	10	ALL TISSUES APPEARED NORMAL.
	6900	DG 20	P	10	KIDNEYS: RIGHT, PELVIS, MODERATE DILATION. ALL OTHER TISSUES APPEARED NORMAL.

P = PREGNANT NP = NOT PREGNANT
DG = DAY OF PRESUMED GESTATION

PROTOCOL 720-002: DEVELOPMENTAL TOXICITY STUDY OF HYAMINE® 1622 IN RATS

TABLE 16 (PAGE 5): NECROPSY OBSERVATIONS - INDIVIDUAL DATA

DOSAGE GROUP DOSAGE (MG/KG/DAY)	RAT NUMBER	DAY OF NECROPSY	PREGNANCY STATUS	DOSAGES ADMINISTERED	OBSERVATIONS
V 170	6901	DG 20	P	10	ALL TISSUES APPEARED NORMAL.
	6902	DG 20	P	10	ALL TISSUES APPEARED NORMAL.
	6903	DG 20	P	10	ALL TISSUES APPEARED NORMAL.
	6904	DG 20	P	10	ALL TISSUES APPEARED NORMAL.
	6905	DG 20	P	10	ALL TISSUES APPEARED NORMAL.
	6906	DG 20	P	10	ALL TISSUES APPEARED NORMAL.
	6907	DG 20	P	10	ALL TISSUES APPEARED NORMAL.
	6908	DG 20	P	10	ALL TISSUES APPEARED NORMAL.
	6909	DG 20	P	10	ALL TISSUES APPEARED NORMAL.
	6910	DG 11	P	6	FOUND DEAD ON DAY 11 OF GESTATION (3 HOURS AND 37 MINUTES AFTER DOSAGE). STOMACH AND INTESTINAL TRACT: GAS-FILLED. KIDNEYS: RIGHT, PELVIS, MODERATE DILATION. SPLEEN: SMALL (0.17 G).a ALL OTHER TISSUES APPEARED NORMAL. UTERINE CONTENTS: 13 IMPLANTATION SITES (13 EMBRYOS).b
	6911	DG 20	P	10	ALL TISSUES APPEARED NORMAL.
	6912	DG 20	P	10	ALL TISSUES APPEARED NORMAL.
	6913	DG 20	P	10	ALL TISSUES APPEARED NORMAL.
	6914	DG 20	P	10	ALL TISSUES APPEARED NORMAL.
	6915	DG 20	P	10	ALL TISSUES APPEARED NORMAL.
	6916	DG 20	P	10	ALL TISSUES APPEARED NORMAL.
	6917	DG 20	P	10	THYMUS: ABSENT. LUNGS: LEFT APICAL, CRANIAL PORTION, DARK RED-BROWN (1.2 CM X 0.5 CM). ALL OTHER TISSUES APPEARED NORMAL.

P = PREGNANT NP = NOT PREGNANT

DG = DAY OF PRESUMED GESTATION

a. Weight recorded after fixation.

b. Appeared normal for their developmental ages at the time maternal death occurred.

PROTOCOL 720-002: DEVELOPMENTAL TOXICITY STUDY OF HYAMINE® 1622 IN RATS

TABLE 16 (PAGE 6): NECROPSY OBSERVATIONS - INDIVIDUAL DATA

DOSAGE GROUP DOSAGE (MG/KG/DAY)	RAT NUMBER	DAY OF NECROPSY	PREGNANCY STATUS	DOSAGES ADMINISTERED	OBSERVATIONS
V (cont.) 170	6918	DG 13	P	7	FOUND DEAD ON DAY 13 OF GESTATION (DEATH OCCURRED OVERNIGHT). STOMACH AND INTESTINAL TRACT: GAS-FILLED. ALL OTHER TISSUES APPEARED NORMAL FOR THE DEGREE OF AUTOLYSIS (MODERATE) PRESENT. UTERINE CONTENTS: 15 IMPLANTATION SITES (15 EMBRYOS). ^a
	6919	DG 20	P	10	ALL TISSUES APPEARED NORMAL.
	6920	DG 20	P	10	ALL TISSUES APPEARED NORMAL.
	6921	DG 16	P	10	FOUND DEAD ON DAY 16 OF GESTATION. LUNGS: ALL LOBES, IRREGULAR SURFACE, MULTIPLE RAISED AREAS. ADRENALS: BILATERAL, LARGE (RIGHT = 0.066 G, LEFT = 0.074 G). DIGESTIVE TRACT: THIN-WALLED AND GAS-FILLED. STOMACH: MULTIPLE BLACK SPOTS (PINPOINT IN SIZE); NO MUCOSAL FOLDS PRESENT. CECUM: FILLED WITH GREEN-BROWN MUCOUS; THIN-WALLED. THYMUS: SMALL (0.009 G). ALL OTHER TISSUES APPEARED NORMAL. UTERINE CONTENTS: 16 IMPLANTATION SITES (16 FETUSES). ^a
	6922	DG 20	P	10	ALL TISSUES APPEARED NORMAL.
	6923	DG 12	P	6	FOUND DEAD ON DAY 12 OF GESTATION (DEATH OCCURRED OVERNIGHT). EXTERNAL OBSERVATION: URINE-STAINED ABDOMINAL FUR. STOMACH: FUNDIC REGION, MUCOSAL SURFACE, NUMEROUS SMALL BLACK SPOTS (LESS THAN 0.1 CM IN DIAMETER). ALL OTHER TISSUES APPEARED NORMAL. UTERINE CONTENTS: 14 IMPLANTATION SITES (14 EMBRYOS). ^b
	6924	DG 20	P	10	ALL TISSUES APPEARED NORMAL.
	6925	DG 20	P	10	ALL TISSUES APPEARED NORMAL.

P = PREGNANT NP = NOT PREGNANT

DG = DAY OF PRESUMED GESTATION

a. Appeared small for their developmental ages at the time maternal death occurred.

b. Appeared normal for their developmental ages at the time maternal death occurred.

PROTOCOL 720-002: DEVELOPMENTAL TOXICITY STUDY OF HYAMINE® 1622 IN RATS

TABLE 17 (PAGE 1): CAESAREAN-SECTIONING OBSERVATIONS - INDIVIDUAL DATA

DOSAGE GROUP I			0(VEHICLE) MG/KG/DAY																	
RAT #	SEX		VIABLE FETUSES			DEAD FETUSES			EARLY RESORPTIONS			LATE RESORPTIONS			IMPLANTATION SITES			CORPORA LUTEA		
	M	F	RIGHT HORN	LEFT HORN	TOTAL	RIGHT HORN	LEFT HORN	TOTAL	RIGHT HORN	LEFT HORN	TOTAL	RIGHT HORN	LEFT HORN	TOTAL	RIGHT HORN	LEFT HORN	TOTAL	RIGHT OVARY	LEFT OVARY	TOTAL
6801	3	11	8	6	14	0	0	0	1	1	2	0	0	0	9	7	16	9	8	17
6802	9	4	6	7	13	0	0	0	0	0	0	0	0	0	6	7	13	8	8	16
6803	4	8	7	5	12	0	0	0	1	1	2	0	0	0	8	6	14	9	7	16
6804	6	9	8	7	15	0	0	0	0	0	0	0	0	0	8	7	15	10	11	21
6805	6	7	7	6	13	0	0	0	0	0	0	0	0	0	7	6	13	8	6	14
6806	9	7	10	6	16	0	0	0	0	1	1	0	0	0	10	7	17	12	7	19
6807	5	9	9	5	14	0	0	0	0	0	0	0	0	0	9	5	14	9	5	14
6808	4	11	6	9	15	0	0	0	0	0	0	0	0	0	6	9	15	6	11	17
6809	8	2	5	5	10	0	0	0	0	1	1	0	0	0	5	6	11	7	8	15
6810	9	7	11	5	16	0	0	0	0	0	0	0	0	0	11	5	16	12	7	19
6811	5	8	4	9	13	0	0	0	0	0	0	0	0	0	4	9	13	5	10	15
6812	7	5	10	2	12	0	0	0	0	0	0	0	0	0	10	2	12	11	2	13
6813	9	7	7	9	16	0	0	0	0	0	0	0	0	0	7	9	16	7	9	16
6814	5	9	8	6	14	0	0	0	0	0	0	0	0	0	8	6	14	10	9	19
6815	8	9	7	10	17	0	0	0	1	1	2	0	0	0	8	11	19	9	11	20
6816	4	10	7	7	14	0	0	0	1	0	1	0	0	0	8	7	15	9	9	18
6817	9	5	2	12	14	0	0	0	0	1	1	0	0	0	2	13	15	3	17	20
6818	9	5	6	8	14	0	0	0	0	0	0	0	0	0	6	8	14	7	10	17
6819	5	8	11	2	13	0	0	0	0	0	0	0	0	0	11	2	13	11	2	13
6820	7	8	5	10	15	0	0	0	0	0	0	0	0	0	5	10	15	6	11	17
6821	10	8	12	6	18	0	0	0	0	0	0	0	0	0	12	6	18	12	8	20
6822	5	11	6	10	16	0	0	0	1	0	1	0	0	0	7	10	17	7	10	17
6823	6	10	13	3	16	0	0	0	0	0	0	0	0	0	13	3	16	13	3	16
6824	10	3	8	5	13	0	0	0	0	1	1	0	0	0	8	6	14	8	7	15
6825	7	9	10	6	16	0	0	0	0	0	0	0	0	0	10	6	16	10	6	16

M = MALE F = FEMALE

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TABLE 17 (PAGE 2): CAESAREAN-SECTIONING OBSERVATIONS - INDIVIDUAL DATA

DOSAGE GROUP II			10 MG/KG/DAY																	
RAT #	SEX		VIABLE FETUSES			DEAD FETUSES			EARLY RESORPTIONS			LATE RESORPTIONS			IMPLANTATION SITES			CORPORA LUTEA		
	M	F	RIGHT HORN	LEFT HORN	TOTAL	RIGHT HORN	LEFT HORN	TOTAL	RIGHT HORN	LEFT HORN	TOTAL	RIGHT HORN	LEFT HORN	TOTAL	RIGHT HORN	LEFT HORN	TOTAL	RIGHT OVARY	LEFT OVARY	TOTAL
3100	9	8	10	7	17	0	0	0	0	0	0	0	0	0	10	7	17	10	7	17
6827	6	7	8	5	13	0	0	0	0	0	0	0	0	0	8	5	13	13	6	19
6828	6	9	9	6	15	0	0	0	0	1	1	0	0	0	9	7	16	9	7	16
6829	4	9	6	7	13	0	0	0	2	0	2	0	0	0	8	7	15	8	8	16
6830	6	9	10	5	15	1	0	1	0	1	1	0	0	0	11	6	17	17	10	27
6831	11	5	7	9	16	0	0	0	0	0	0	0	0	0	7	9	16	8	11	19
6832	7	8	9	6	15	0	0	0	0	0	0	0	0	0	9	6	15	11	6	17
6833	7	6	5	8	13	0	0	0	0	0	0	0	0	0	5	8	13	6	11	17
6834	8	5	3	10	13	0	0	0	0	0	0	0	0	0	3	10	13	3	10	13
6835	SACRIFICED ON DAY 20 OF GESTATION; DAM IN THE PROCESS OF DELIVERY																			
6836	7	8	10	5	15	0	0	0	0	0	0	0	0	0	10	5	15	10	5	15
6837	7	6	6	7	13	0	0	0	0	1	1	0	0	0	6	8	14	7	9	16
6838	9	5	5	9	14	0	0	0	1	0	1	0	0	0	6	9	15	6	9	15
6839	12	5	8	9	17	0	0	0	0	0	0	0	0	0	8	9	17	10	10	20
6840	7	3	6	4	10	0	0	0	1	0	1	0	0	0	7	4	11	8	4	12
6841	6	7	7	6	13	0	0	0	2	0	2	0	0	0	9	6	15	10	8	18
6842	10	5	8	7	15	0	0	0	0	0	0	0	0	0	8	7	15	8	7	15
6843	5	5	5	5	10	0	0	0	1	2	3	0	0	0	6	7	13	7	7	14
6844	7	6	10	3	13	0	0	0	1	2	3	0	0	0	11	5	16	11	7	18
6845	8	6	8	6	14	0	0	0	2	0	2	0	0	0	10	6	16	11	6	17
6846	7	9	9	7	16	0	0	0	0	0	0	0	0	0	9	7	16	12	8	20
6847	NOT PREGNANT																			
6848	9	7	6	10	16	0	0	0	0	0	0	0	0	0	6	10	16	6	12	18
6849	6	9	8	7	15	0	0	0	2	0	2	0	0	0	10	7	17	11	9	20
6850	5	9	7	7	14	0	0	0	0	0	0	0	0	0	7	7	14	7	8	15

M = MALE F = FEMALE

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TABLE 17 (PAGE 3): CAESAREAN-SECTIONING OBSERVATIONS - INDIVIDUAL DATA

DOSAGE GROUP III					30 MG/KG/DAY															
RAT #	VIABLE FETUSES				DEAD FETUSES			EARLY RESORPTIONS		LATE RESORPTIONS		IMPLANTATION SITES				CORPORA LUTEA				
	SEX		RIGHT LEFT		RIGHT LEFT		RIGHT LEFT		RIGHT LEFT		RIGHT LEFT		RIGHT LEFT		RIGHT LEFT		RIGHT LEFT			
	M	F	HORN	TOTAL	HORN	TOTAL	HORN	TOTAL	HORN	TOTAL	HORN	TOTAL	HORN	TOTAL	HORN	TOTAL	Ovary	TOTAL		
6851	NOT PREGNANT																			
6852	9	6	6	9	15	0	0	0	3	0	3	0	0	0	9	9	18	9	9	18
6853	8	8	7	9	16	0	0	0	0	0	0	0	0	0	7	9	16	7	9	16
6854	7	7	9	5	14	0	0	0	0	0	0	0	0	0	9	5	14	9	6	15
6855	6	7	7	6	13	0	0	0	0	0	0	0	0	0	7	6	13	8	7	15
6856	7	9	9	7	16	0	0	0	0	0	0	0	0	0	9	7	16	11	8	19
6857	6	5	5	6	11	0	0	0	0	3	3	0	0	0	5	9	14	5	9	14
6858	7	8	10	5	15	0	0	0	1	0	1	0	0	0	11	5	16	12	6	18
6859	8	6	9	5	14	0	0	0	1	1	2	0	0	0	10	6	16	10	7	17
6860	6	10	9	7	16	0	0	0	0	0	0	0	0	0	9	7	16	12	7	19
6861	8	5	4	9	13	0	0	0	0	1	1	0	0	0	4	10	14	5	10	15
6862	6	8	8	6	14	0	0	0	0	1	1	0	0	0	8	7	15	9	9	18
6863	9	7	11	5	16	0	0	0	0	0	0	0	0	0	11	5	16	11	5	16
6864	7	9	8	8	16	0	0	0	0	0	0	0	0	0	8	8	16	8	9	17
6865	8	6	7	7	14	0	0	0	0	0	0	0	0	0	7	7	14	9	9	18
6866	9	6	10	5	15	0	0	0	0	1	1	0	0	0	10	6	16	12	7	19
6867	9	5	9	5	14	0	0	0	0	0	0	0	0	0	9	5	14	10	6	16
6868	9	3	5	7	12	0	0	0	0	0	0	0	0	0	5	7	12	10	10	20
6869	7	8	6	9	15	0	0	0	0	0	0	0	0	0	6	9	15	7	9	16
6870	6	4	7	3	10	0	0	0	1	0	1	0	0	0	8	3	11	8	3	11
6871	7	8	7	8	15	0	0	0	1	0	1	0	0	0	8	8	16	10	8	18
6872	7	6	6	7	13	0	0	0	0	1	1	0	0	0	6	8	14	6	9	15
6873	8	7	7	8	15	0	0	0	0	0	0	0	0	0	7	8	15	7	8	15
6874	8	7	5	10	15	0	0	0	0	1	1	0	0	0	5	11	16	5	11	16
6875	11	4	6	9	15	0	0	0	1	0	1	0	0	0	7	9	16	8	10	18

M = MALE F = FEMALE

PROTOCOL 720-002: DEVELOPMENTAL TOXICITY STUDY OF HYAMINE® 1622 IN RATS

TABLE 17 (PAGE 4): CAESAREAN-SECTIONING OBSERVATIONS - INDIVIDUAL DATA

DOSAGE GROUP IV			100 MG/KG/DAY																	
RAT #	SEX		VIABLE FETUSES			DEAD FETUSES			EARLY RESORPTIONS			LATE RESORPTIONS			IMPLANTATION SITES			CORPORA LUTEA		
	M	F	RIGHT LEFT		TOTAL	RIGHT LEFT		TOTAL	RIGHT LEFT		TOTAL	RIGHT LEFT		TOTAL	RIGHT LEFT		TOTAL	RIGHT LEFT		TOTAL
			HORN			HORN			HORN			HORN			HORN			OVARY		
6876	7	8	8	7	15	0	0	0	0	1	1	0	0	0	8	8	16	9	8	17
6877	4	9	4	9	13	0	0	0	1	0	1	0	0	0	5	9	14	6	9	15
6878	8	6	11	3	14	0	0	0	0	0	0	0	0	0	11	3	14	12	4	16
6879	6	7	6	7	13	0	0	0	2	2	4	0	0	0	8	9	17	8	10	18
6880	9	5	6	8	14	0	0	0	0	0	0	0	0	0	6	8	14	7	9	16
6881	NOT PREGNANT																			
6882	6	5	6	5	11	0	0	0	0	2	2	0	0	0	6	7	13	7	8	15
6883	4	10	7	7	14	0	0	0	0	0	0	0	0	0	7	7	14	8	8	16
6884	9	5	5	9	14	0	0	0	2	0	2	0	0	0	7	9	16	7	10	17
6885	2	7	2	7	9	0	0	0	3	1	4	0	0	0	5	8	13	6	9	15
6886	7	7	9	5	14	0	0	0	0	2	2	0	0	0	9	7	16	9	8	17
6887	6	6	5	7	12	0	0	0	1	0	1	0	0	0	6	7	13	8	8	16
6888	10	7	9	8	17	0	0	0	0	0	0	0	0	0	9	8	17	11	9	20
6889	12	4	9	7	16	0	0	0	0	0	0	0	0	0	9	7	16	9	8	17
6890	8	5	6	7	13	0	0	0	0	0	0	0	0	0	6	7	13	7	8	15
6891	7	7	9	5	14	0	0	0	0	1	1	0	1	1	9	7	16	9	7	16
6892	7	9	5	11	16	0	0	0	1	0	1	0	0	0	6	11	17	8	12	20
6893	8	5	7	6	13	0	0	0	1	0	1	0	0	0	8	6	14	9	6	15
6894	4	10	6	8	14	0	0	0	2	0	2	0	0	0	8	8	16	8	8	16
6895	6	9	10	5	15	0	0	0	1	0	1	1	0	1	12	5	17	12	5	17
6896	9	5	7	7	14	0	0	0	0	2	2	0	0	0	7	9	16	9	9	18
6897	5	10	5	10	15	0	0	0	1	2	3	0	0	0	6	12	18	6	12	18
6898	10	6	7	9	16	0	0	0	0	0	0	0	0	0	7	9	16	9	11	20
6899	5	9	8	6	14	0	0	0	1	0	1	0	0	0	9	6	15	9	6	15
6900	7	8	11	4	15	0	0	0	0	1	1	0	0	0	11	5	16	13	5	18

M = MALE F = FEMALE

PROTOCOL 720-002: DEVELOPMENTAL TOXICITY STUDY OF HYAMINE® 1622 IN RATS

TABLE 17 (PAGE 5): CAESAREAN-SECTIONING OBSERVATIONS - INDIVIDUAL DATA

DOSAGE GROUP V			170 MG/KG/DAY																		
RAT #	SEX		VIABLE FETUSES			DEAD FETUSES			EARLY RESORPTIONS			LATE RESORPTIONS			IMPLANTATION SITES			CORPORA LUTEA			
	M	F	RIGHT LEFT		TOTAL	RIGHT LEFT		TOTAL	RIGHT LEFT		TOTAL	RIGHT LEFT		TOTAL	RIGHT LEFT		TOTAL	RIGHT LEFT		TOTAL	
			HORN			HORN			HORN			HORN			HORN			OVARY			
6901	7	5	9	3	12	0	0	0	0	0	0	0	0	0	9	3	12	9	4	13	
6902	7	7	9	5	14	0	0	0	0	0	0	0	0	0	9	5	14	10	7	17	
6903	9	9	10	8	18	0	0	0	0	0	0	0	0	0	10	8	18	10	8	18	
6904	7	8	9	6	15	0	0	0	0	0	0	0	0	0	9	6	15	12	7	19	
6905	4	13	9	8	17	0	0	0	0	0	0	0	0	0	9	8	17	11	9	20	
6906	7	7	7	7	14	0	0	0	0	2	2	0	0	0	7	9	16	7	10	17	
6907	4	1	4	1	5	0	0	0	3	6	9	0	0	0	7	7	14	8	7	15	
6908	7	4	6	5	11	0	0	0	0	2	2	0	0	0	6	7	13	11	8	19	
6909	6	6	5	7	12	0	0	0	1	0	1	0	0	0	6	7	13	7	9	16	
6910	FOUND DEAD ON DAY 11 OF GESTATION																				
6911	7	6	6	7	13	0	0	0	2	0	2	0	0	0	8	7	15	9	8	17	
6912	7	3	10	0	10	0	0	0	0	0	0	0	0	0	10	0	10	11	4	15	
6913	8	6	9	5	14	0	0	0	0	0	0	0	0	0	9	5	14	9	6	15	
6914	6	8	5	9	14	0	0	0	0	2	2	0	0	0	5	11	16	6	12	18	
6915	9	6	6	9	15	0	0	0	1	0	1	0	0	0	7	9	16	7	9	16	
6916	8	5	8	5	13	0	0	0	1	0	1	0	0	0	9	5	14	9	7	16	
6917	4	4	6	2	8	0	0	0	2	0	2	0	0	0	8	2	10	9	8	17	
6918	FOUND DEAD ON DAY 13 OF GESTATION																				
6919	3	3	1	5	6	0	0	0	0	0	0	0	0	0	1	5	6	2	5	7	
6920	6	8	8	6	14	0	0	0	0	0	0	0	0	0	8	6	14	9	7	16	
6921	FOUND DEAD ON DAY 16 OF GESTATION																				
6922	7	7	8	6	14	0	0	0	0	0	0	0	0	0	8	6	14	10	9	19	
6923	FOUND DEAD ON DAY 12 OF GESTATION																				
6924	5	8	9	4	13	0	0	0	2	0	2	0	0	0	11	4	15	11	9	20	
6925	10	8	7	11	18	0	0	0	0	0	0	0	0	0	7	11	18	7	12	19	

M = MALE F = FEMALE

PROTOCOL 720-002: DEVELOPMENTAL TOXICITY STUDY OF HYAMINE® 1622 IN RATS

TABLE 18 (PAGE 1): LITTER OBSERVATIONS (CAESAREAN-DELIVERED FETUSES) - INDIVIDUAL DATA

DOSAGE GROUP I			0(VEHICLE) MG/KG/DAY						
RAT #	NUMBER OF LIVE FETUSES			AVERAGE FETAL BODY WEIGHT (G)			---- CONCEPTUSES ----- DEAD OR RESORBED		
	MALE	FEMALE	TOTAL	MALE	FEMALE	TOTAL ^a	N	N	%
6801	3	11	14	3.04	2.79	2.84	16	2	12.5
6802	9	4	13	3.61	3.36	3.53	13	0	0.0
6803	4	8	12	3.70	3.40	3.50	14	2	14.3
6804	6	9	15	3.73	3.54	3.62	15	0	0.0
6805	6	7	13	3.64	3.54	3.59	13	0	0.0
6806	9	7	16	3.46	3.26	3.37	17	1	5.9
6807	5	9	14	3.28	3.39	3.35	14	0	0.0
6808	4	11	15	3.45	3.21	3.28	15	0	0.0
6809	8	2	10	3.50	3.70	3.54	11	1	9.1
6810	9	7	16	3.45	3.18	3.33	16	0	0.0
6811	5	8	13	3.20	3.04	3.10	13	0	0.0
6812	7	5	12	3.81	3.48	3.68	12	0	0.0
6813	9	7	16	3.47	3.32	3.40	16	0	0.0
6814	5	9	14	3.42	3.11	3.22	14	0	0.0
6815	8	9	17	3.54	3.29	3.40	19	2	10.5
6816	4	10	14	3.64	3.39	3.46	15	1	6.7
6817	9	5	14	3.31	3.22	3.28	15	1	6.7
6818	9	5	14	3.21	3.31	3.24	14	0	0.0
6819	5	8	13	3.01	2.94	2.96	13	0	0.0
6820	7	8	15	3.57	3.44	3.50	15	0	0.0
6821	10	8	18	3.52	3.21	3.38	18	0	0.0
6822	5	11	16	3.83	3.68	3.73	17	1	5.9
6823	6	10	16	3.41	3.35	3.37	16	0	0.0
6824	10	3	13	3.31	3.20	3.28	14	1	7.1
6825	7	9	16	3.43	3.32	3.37	16	0	0.0

a. TOTAL = SUM OF FETAL WEIGHTS/NUMBER OF LIVE FETUSES.

PROTOCOL 720-002: DEVELOPMENTAL TOXICITY STUDY OF HYAMINE® 1622 IN RATS

TABLE 18 (PAGE 2): LITTER OBSERVATIONS (CAESAREAN-DELIVERED FETUSES) - INDIVIDUAL DATA

DOSAGE GROUP II			10 MG/KG/DAY						
RAT #	NUMBER OF LIVE FETUSES			AVERAGE FETAL BODY WEIGHT (G)			---- CONCEPTUSES ---- DEAD OR RESORBED		
	MALE	FEMALE	TOTAL	MALE	FEMALE	TOTAL ^a	N	N	%
3100	9	8	17	3.46	3.28	3.38	17	0	0.0
6827	6	7	13	3.55	3.32	3.43	13	0	0.0
6828	6	9	15	3.74	3.37	3.52	16	1	6.2
6829	4	9	13	2.88	2.73	2.77	15	2	13.3
6830	6	9	15	3.25	3.16	3.20	17	2	11.8
6831	11	5	16	3.66	3.55	3.62	16	0	0.0
6832	7	8	15	3.85	3.63	3.73	15	0	0.0
6833	7	6	13	3.43	3.20	3.33	13	0	0.0
6834	8	5	13	3.63	3.60	3.62	13	0	0.0
6835	SACRIFICED ON DAY 20 OF GESTATION; DAM IN THE PROCESS OF DELIVERY								
6836	7	8	15	3.52	3.14	3.32	15	0	0.0
6837	7	6	13	3.55	3.11	3.34	14	1	7.1
6838	9	5	14	3.10	2.82	3.00	15	1	6.7
6839	12	5	17	3.75	3.51	3.68	17	0	0.0
6840	7	3	10	3.34	3.16	3.29	11	1	9.1
6841	6	7	13	3.12	2.84	2.96	15	2	13.3
6842	10	5	15	3.34	3.06	3.25	15	0	0.0
6843	5	5	10	3.73	3.35	3.54	13	3	23.1
6844	7	6	13	3.32	3.27	3.30	16	3	18.8
6845	8	6	14	3.60	3.28	3.46	16	2	12.5
6846	7	9	16	3.72	3.50	3.60	16	0	0.0
6847	NOT PREGNANT								
6848	9	7	16	3.01	2.98	3.00	16	0	0.0
6849	6	9	15	3.48	3.28	3.36	17	2	11.8
6850	5	9	14	3.56	3.48	3.51	14	0	0.0

^a. TOTAL = SUM OF FETAL WEIGHTS/NUMBER OF LIVE FETUSES.

PROTOCOL 720-002: DEVELOPMENTAL TOXICITY STUDY OF HYAMINE® 1622 IN RATS

TABLE 18 (PAGE 3): LITTER OBSERVATIONS (CAESAREAN-DELIVERED FETUSES) - INDIVIDUAL DATA

DOSAGE GROUP III				30 MG/KG/DAY					
RAT #	NUMBER OF LIVE FETUSES			AVERAGE FETAL BODY WEIGHT (G)			---- CONCEPTUSES ----- DEAD OR RESORBED		
	MALE	FEMALE	TOTAL	MALE	FEMALE	TOTAL ^a	N	N	%
6851	NOT PREGNANT								
6852	9	6	15	3.52	3.22	3.40	18	3	16.7
6853	8	8	16	3.55	3.25	3.40	16	0	0.0
6854	7	7	14	3.19	2.92	3.06	14	0	0.0
6855	6	7	13	3.40	3.10	3.24	13	0	0.0
6856	7	9	16	3.44	3.19	3.30	16	0	0.0
6857	6	5	11	3.33	3.11	3.23	14	3	21.4
6858	7	8	15	3.29	3.14	3.21	16	1	6.2
6859	8	6	14	3.54	3.43	3.49	16	2	12.5
6860	6	10	16	4.10	3.87	3.96	16	0	0.0
6861	8	5	13	3.31	3.07	3.22	14	1	7.1
6862	6	8	14	3.64	3.42	3.52	15	1	6.7
6863	9	7	16	3.11	2.82	2.98	16	0	0.0
6864	7	9	16	3.35	2.91	3.10	16	0	0.0
6865	8	6	14	3.63	3.45	3.55	14	0	0.0
6866	9	6	15	3.54	3.35	3.46	16	1	6.2
6867	9	5	14	3.37	3.02	3.25	14	0	0.0
6868	9	3	12	3.79	3.30	3.66	12	0	0.0
6869	7	8	15	3.18	2.99	3.08	15	0	0.0
6870	6	4	10	3.22	3.09	3.17	11	1	9.1
6871	7	8	15	3.52	3.26	3.38	16	1	6.2
6872	7	6	13	3.46	3.08	3.28	14	1	7.1
6873	8	7	15	3.36	3.10	3.24	15	0	0.0
6874	8	7	15	3.11	2.86	2.99	16	1	6.2
6875	11	4	15	3.34	3.47	3.38	16	1	6.2

^a. TOTAL = SUM OF FETAL WEIGHTS/NUMBER OF LIVE FETUSES.

PROTOCOL 720-002: DEVELOPMENTAL TOXICITY STUDY OF HYAMINE® 1622 IN RATS

TABLE 18 (PAGE 4): LITTER OBSERVATIONS (CAESAREAN-DELIVERED FETUSES) - INDIVIDUAL DATA

DOSAGE GROUP IV				100 MG/KG/DAY					
RAT #	NUMBER OF LIVE FETUSES			AVERAGE FETAL BODY WEIGHT (G)			----- CONCEPTUSES ----- DEAD OR RESORBED		
	MALE	FEMALE	TOTAL	MALE	FEMALE	TOTAL ^a	N	N	%
6876	7	8	15	3.35	3.08	3.21	16	1	6.2
6877	4	9	13	3.47	3.27	3.33	14	1	7.1
6878	8	6	14	3.93	3.48	3.74	14	0	0.0
6879	6	7	13	3.09	2.85	2.96	17	4	23.5
6880	9	5	14	3.34	3.21	3.30	14	0	0.0
6881	NOT PREGNANT								
6882	6	5	11	3.34	3.21	3.28	13	2	15.4
6883	4	10	14	3.45	3.26	3.31	14	0	0.0
6884	9	5	14	3.54	3.10	3.38	16	2	12.5
6885	2	7	9	2.84	3.54	3.38	13	4	30.8
6886	7	7	14	3.08	3.21	3.14	16	2	12.5
6887	6	6	12	3.50	3.64	3.57	13	1	7.7
6888	10	7	17	3.20	2.66	2.97	17	0	0.0
6889	12	4	16	3.22	2.81	3.12	16	0	0.0
6890	8	5	13	3.62	3.35	3.52	13	0	0.0
6891	7	7	14	3.07	2.96	3.02	16	2	12.5
6892	7	9	16	3.50	3.32	3.40	17	1	5.9
6893	8	5	13	3.05	3.04	3.04	14	1	7.1
6894	4	10	14	3.60	3.20	3.32	16	2	12.5
6895	6	9	15	3.08	3.09	3.08	17	2	11.8
6896	9	5	14	3.37	3.22	3.32	16	2	12.5
6897	5	10	15	2.74	2.36	2.48	18	3	16.7
6898	10	6	16	3.75	3.58	3.68	16	0	0.0
6899	5	9	14	3.16	3.13	3.14	15	1	6.7
6900	7	8	15	3.00	2.92	2.96	16	1	6.2

a. TOTAL = SUM OF FETAL WEIGHTS/NUMBER OF LIVE FETUSES.

PROTOCOL 720-002: DEVELOPMENTAL TOXICITY STUDY OF HYAMINE® 1622 IN RATS

TABLE 18 (PAGE 5): LITTER OBSERVATIONS (CAESAREAN-DELIVERED FETUSES) - INDIVIDUAL DATA

DOSAGE GROUP V			170 MG/KG/DAY						
RAT #	NUMBER OF LIVE FETUSES			AVERAGE FETAL BODY WEIGHT (G)			---- CONCEPTUSES ----- DEAD OR RESORBED		
	MALE	FEMALE	TOTAL	MALE	FEMALE	TOTAL ^a	N	N	%
6901	7	5	12	2.98	2.57	2.80	12	0	0.0
6902	7	7	14	3.73	3.60	3.66	14	0	0.0
6903	9	9	18	3.87	3.66	3.76	18	0	0.0
6904	7	8	15	3.43	3.37	3.40	15	0	0.0
6905	4	13	17	3.69	3.65	3.66	17	0	0.0
6906	7	7	14	3.74	3.50	3.62	16	2	12.5
6907	4	1	5	3.80	3.63	3.76	14	9	64.3
6908	7	4	11	3.24	3.28	3.26	13	2	15.4
6909	6	6	12	3.46	3.38	3.42	13	1	7.7
6910	FOUND DEAD ON DAY 11 OF GESTATION								
6911	7	6	13	3.53	3.27	3.41	15	2	13.3
6912	7	3	10	3.43	3.31	3.40	10	0	0.0
6913	8	6	14	2.81	2.64	2.73	14	0	0.0
6914	6	8	14	3.65	3.25	3.42	16	2	12.5
6915	9	6	15	2.92	2.90	2.91	16	1	6.2
6916	8	5	13	3.67	3.64	3.66	14	1	7.1
6917	4	4	8	2.52	2.34	2.43	10	2	20.0
6918	FOUND DEAD ON DAY 13 OF GESTATION								
6919	3	3	6	2.99	2.98	2.98	6	0	0.0
6920	6	8	14	3.32	3.31	3.31	14	0	0.0
6921	FOUND DEAD ON DAY 16 OF GESTATION								
6922	7	7	14	3.07	3.06	3.07	14	0	0.0
6923	FOUND DEAD ON DAY 12 OF GESTATION								
6924	5	8	13	2.99	2.87	2.92	15	2	13.3
6925	10	8	18	3.29	2.83	3.08	18	0	0.0

a. TOTAL = SUM OF FETAL WEIGHTS/NUMBER OF LIVE FETUSES.

PROTOCOL 720-002: DEVELOPMENTAL TOXICITY STUDY OF HYAMINE® 1622 IN RATS

TABLE 19 (PAGE 1): FETAL SEX, VITAL STATUS AND BODY WEIGHT - INDIVIDUAL DATA

DOSAGE GROUP I		0 (VEHICLE) MG/KG/DAY																						
FETUS #		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23
RAT #	CLS																							
6801	9/ 8	FA	FA	FA	FA	E	FA	FA	MA	FA / FA	MA	MA	FA	FA	E	FA								
		1.77	2.43	2.93	2.80		3.02	2.66	3.17	2.96	3.04	2.86	3.08	3.07	2.99		3.03							
6802	8/ 8	MA	MA	MA	MA	MA	FA / FA	MA	MA	MA	FA	MA	FA											
		3.55	3.42	3.78	3.40	3.56	3.47	3.40	3.81	3.48	3.61	3.27	3.85	3.29										
6803	9/ 7	FA	MA	MA	E	FA	FA	FA	FA / MA	FA	E	FA	FA	MA										
		3.41	3.71	3.79		3.62	3.16	3.20	3.64	3.73	3.49		3.45	3.20	3.59									
6804	10/11	FA	MA	MA	FA	FA	FA	MA	MA / FA	FA	MA	MA	FA	FA	FA									
		3.08	3.57	3.58	3.68	3.61	3.32	3.99	3.90	3.42	3.64	3.57	3.75	3.59	3.62	3.95								
6805	8/ 6	FA	FA	FA	MA	MA	MA	MA / FA	MA	FA	FA	MA	FA	MA	FA									
		3.63	3.63	3.64	3.42	3.64	3.81	3.61	3.33	3.82	3.77	3.43	3.57	3.33										
6806	12/ 7	MA	MA	MA	MA	MA	FA	FA	MA	FA	FA / MA	MA	FA	FA	FA	E	MA							
		3.30	3.65	3.38	3.20	3.36	3.25	2.74	3.43	3.31	3.27	3.27	3.83	3.21	3.42	3.62		3.70						
6807	9/ 5	MA	MA	MA	FA	FA	FA	FA	FA / FA	FA	MA	MA	FA	MA	FA									
		3.19	3.41	3.27	3.42	3.22	3.53	3.41	3.33	3.60	3.14	3.26	3.32	3.21	3.59									
6808	6/11	FA	FA	FA	FA	MA	FA / MA	MA	MA	FA	FA	FA	FA	FA	FA									
		3.27	3.41	3.35	3.25	3.39	3.31	3.39	3.38	3.64	2.84	3.28	3.20	3.17	3.08	3.19								
6809	7/ 8	MA	MA	MA	MA	MA / MA	E	MA	MA	FA	FA													
		3.19	3.38	3.42	3.32	3.86	3.07		3.73	4.04	3.72	3.68												
6810	12/ 7	FA	MA	MA	MA	FA	FA	FA	FA	MA	MA	MA / FA	MA	MA	FA	MA								
		2.42	3.49	3.39	3.51	2.92	3.51	3.55	3.62	3.92	3.69	3.35	2.90	3.41	2.90	3.33	3.37							
6811	5/10	FA	FA	MA	FA / FA	MA	FA	FA	FA	FA	FA	MA	MA	MA										
		3.35	3.42	3.57	3.19	2.70	2.97	2.88	3.03	3.06	2.66	3.12	2.99	3.35										
6812	11/ 2	FA	FA	MA	FA	MA	MA	MA	MA	MA / FA	FA													
		3.33	3.55	3.85	3.43	4.10	3.76	3.81	3.49	3.58	4.11	3.36	3.74											
6813	7/ 9	MA	MA	FA	MA	FA	FA	FA / MA	FA	MA	MA	MA	FA	MA	FA	MA	MA							
		3.32	3.40	3.20	3.56	3.32	3.09	3.47	3.55	3.42	3.45	3.47	3.27	3.70	3.28	3.48	3.49							

M = MALE F = FEMALE A = ALIVE D = DEAD E = EARLY RESORPTION L = LATE RESORPTION */* DENOTES POSITION OF CERVIX
 CLS = CORPORA LUTEA/OVARY FETAL BODY WEIGHTS WERE RECORDED IN GRAMS (G).

PROTOCOL 720-002: DEVELOPMENTAL TOXICITY STUDY OF HYAMINE® 1622 IN RATS

TABLE 19 (PAGE 2): FETAL SEX, VITAL STATUS AND BODY WEIGHT - INDIVIDUAL DATA

DOSAGE GROUP I		0(VEHICLE) MG/KG/DAY																						
FETUS #		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23
RAT #	CLs																							
6814	10/ 9	FA	FA	MA	MA	FA	FA	MA	FA / MA	FA	FA	FA	FA	FA	MA									
		2.35	3.30	3.66	3.04	3.30	3.23	3.11	3.28	3.86	3.13	2.89	3.07	3.45	3.43									
6815	9/11	MA	MA	E	MA	FA	FA	MA	MA / FA	FA	FA	MA	FA	MA	E	FA	FA	FA	MA					
		3.49	3.13		3.67	3.49	3.56	3.64	3.67	3.35	3.21	3.45	3.39	3.42	3.57		3.72	3.25	2.14	3.73				
6816	9/ 9	FA	FA	FA	E	FA	FA	FA	FA / FA	MA	MA	FA	FA	MA	MA									
		2.72	3.28	3.26		3.59	3.33	3.52	3.44	3.63	3.53	3.65	3.61	3.49	3.67	3.71								
6817	3/17	FA	MA / FA	FA	MA	FA	MA	MA	MA	MA	E	MA	FA	MA	MA									
		3.42	3.49	3.12	3.48	3.46	3.02	3.19	3.47	3.01	3.57		3.47	3.07	2.85	3.28								
6818	7/10	MA	MA	MA	MA	MA	FA / FA	FA	FA	MA	FA	MA	MA	MA	MA									
		3.19	3.56	3.00	3.21	3.38	3.55	3.41	3.20	3.25	3.34	3.14	2.81	2.81	3.58									
6819	11/ 2	MA	FA	FA	FA	FA	FA	FA	MA	FA	FA	MA / MA	MA											
		2.40	2.80	2.93	3.03	3.00	3.09	2.98	3.11	2.76	2.89	2.96	3.44	3.12										
6820	6/11	MA	FA	FA	FA	MA / MA	MA	FA	FA	MA	FA	MA	MA	FA	FA									
		3.20	3.36	3.70	3.57	3.64	3.64	3.63	3.41	3.20	3.47	3.62	3.70	3.70	3.14	3.56								
6821	12/ 8	MA	FA	MA	FA	MA	MA	MA	FA	FA	MA	MA	FA / FA	MA	FA	MA	MA	FA						
		3.44	2.80	3.52	2.92	3.55	3.87	3.29	3.22	3.20	3.29	3.62	3.29	3.32	3.62	3.38	3.64	3.41	3.52					
6822	7/10	FA	E	FA	FA	FA	MA	FA / MA	FA	FA	FA	FA	FA	MA	FA	MA	FA	MA						
		3.54		3.71	3.68	3.64	3.42	3.53	3.90	3.60	3.88	3.69	3.91	3.69	3.44	4.13	3.85	4.03						
6823	13/ 3	FA	MA	MA	MA	FA	MA	FA	FA	FA	MA	FA	FA	FA	FA / FA	MA	FA							
		3.28	3.38	3.34	3.57	3.51	3.45	3.66	3.26	3.32	3.41	3.15	3.53	3.22	3.27	3.29	3.28							
6824	8/ 7	MA	FA	FA	MA	MA	FA	MA	MA / MA	MA	MA	MA	MA	E										
		3.61	3.35	3.14	3.43	3.22	3.10	3.24	3.59	3.17	3.03	3.09	3.48	3.21										
6825	10/ 6	MA	FA	MA	MA	MA	FA	FA	MA	FA	FA / FA	FA	FA	FA	MA	MA	FA							
		3.21	3.16	3.49	3.18	3.63	3.34	3.32	3.47	3.12	3.11	3.35	3.63	3.38	3.73	3.33	3.51							

M = MALE F = FEMALE A = ALIVE D = DEAD E = EARLY RESORPTION L = LATE RESORPTION "/" DENOTES POSITION OF CERVIX
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PROTOCOL 720-002: DEVELOPMENTAL TOXICITY STUDY OF HYAMINE® 1622 IN RATS

TABLE 19 (PAGE 3): FETAL SEX, VITAL STATUS AND BODY WEIGHT - INDIVIDUAL DATA

DOSAGE GROUP II		10 MG/KG/DAY																						
FETUS #		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23
RAT #	CLs																							
3100	10/ 7	MA	MA	MA	MA	MA	FA	FA	MA	MA	FA / MA	FA	FA	FA	FA	MA	FA	FA						
		3.20	3.33	3.68	3.78	3.38	3.22	3.15	3.31	3.30	3.12	3.61	3.51	3.30	3.41	3.54	3.17	3.38						
6827	13/ 6	FA	MA	MA	FA	MA	FA	MA	FA / FA	FA	MA	FA	MA											
		3.58	3.19	3.42	3.47	3.73	2.75	3.83	3.62	3.02	3.51	3.56	3.30	3.58										
6828	9/ 7	FA	MA	FA	FA	FA	FA	MA	FA / E	FA	MA	MA	MA	MA	MA	MA	FA							
		3.32	3.27	2.93	3.49	3.32	3.55	3.29	3.60	3.51		3.53	3.95	3.79	3.60	4.21	3.43							
6829	8/ 8	FA	FA	FA	MA	E	FA	MA	E / FA	MA	FA	FA	FA	FA	MA	FA								
		2.73	2.92	2.75	2.72		2.92	2.67		2.59	3.04	2.58	2.71	2.51	3.08	2.83								
6830	17/10	FA	FA	FA	FA	MA	MA	FA	MA	MD	MA	MA / FA	FA	FA	E	MA	FA	FA						
		3.07	3.13	2.90	3.06	3.25	3.12	3.05	3.29		3.11	3.11	3.29	3.30		3.60	3.57	3.12						
6831	8/11	FA	MA	MA	MA	MA	MA	MA / MA	MA	MA	MA	MA	FA	FA	FA	FA	MA							
		3.62	3.57	3.69	4.09	3.44	3.91	3.65	3.33	3.80	3.35	3.56	3.54	3.58	3.45	3.54	3.87							
6832	11/ 6	MA	MA	FA	MA	FA	MA	MA	FA	MA / FA	MA	FA	FA	FA	FA	FA	FA							
		3.63	3.90	3.82	3.98	3.39	4.00	3.73	3.81	3.77	3.54	3.96	3.49	3.53	3.83	3.60								
6833	6/11	MA	MA	MA	FA	FA / FA	MA	MA	FA	FA	MA	MA	FA											
		3.73	3.64	3.49	3.38	3.28	3.25	3.41	3.18	2.88	3.44	3.39	3.17	3.00										
6834	3/10	MA	MA	MA / FA	FA	FA	MA	MA	MA	MA	FA	FA	MA	FA										
		3.76	3.50	3.76	3.41	3.49	3.37	3.60	3.84	3.51	3.79	3.71	3.67	3.60										
6835		SACRIFICED ON DAY 20 OF GESTATION; DAM IN THE PROCESS OF DELIVERY																						
6836	10/ 5	FA	FA	MA	FA	MA	MA	FA	FA	FA	MA / MA	FA	MA	FA	MA									
		2.84	3.32	3.55	3.09	3.18	3.53	3.11	3.49	3.19	3.67	3.80	2.60	3.35	3.49	3.55								
6837	7/ 9	FA	FA	MA	FA	MA	FA / MA	MA	FA	E	MA	MA	MA	FA										
		3.19	3.47	3.87	3.21	3.51	2.41	3.62	3.64	3.29		3.66	3.33	3.20	3.09									
6838	6/ 9	E	FA	FA	MA	MA	MA / FA	MA	FA	MA	MA	MA	MA	MA	FA	MA								
		3.02	2.85	3.35	2.96	3.10	2.90	3.16	2.83	3.09	3.06	3.22	3.07	2.49	2.86									

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PROTOCOL 720-002: DEVELOPMENTAL TOXICITY STUDY OF HYAMINE® 1622 IN RATS

TABLE 19 (PAGE 4): FETAL SEX, VITAL STATUS AND BODY WEIGHT - INDIVIDUAL DATA

DOSAGE GROUP II		10 MG/KG/DAY																						
FETUS #		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23
RAT #	CLs																							
6839	10/10	MA	FA	MA	MA	MA	MA	MA	FA / MA	FA	MA	FA	MA	FA	MA	MA	MA	MA						
		3.82	3.31	3.91	3.47	3.98	3.94	3.70	3.72	3.36	3.29	3.66	3.58	3.75	3.67	3.71	3.86	3.83						
6840	8/ 4	FA	MA	MA	MA	FA	E	MA / FA	MA	MA	MA													
		3.06	3.53	3.34	3.56	3.29		3.42	3.14	3.34	3.27	2.93												
6841	10/ 8	FA	MA	MA	FA	FA	E	MA	MA	E / FA	FA	FA	FA	FA	MA	MA								
		2.69	3.24	2.59	2.68	2.60		3.06	3.30		3.15	2.55	3.09	3.09	3.16	3.34								
6842	8/ 7	MA	MA	MA	FA	FA	MA	MA	FA / FA	FA	MA	MA	MA	MA	MA	FA	MA							
		2.50	3.33	3.27	2.41	3.20	3.44	3.41	3.11	3.19	3.17	3.44	3.62	3.53	3.40	3.72								
6843	7/ 7	MA	MA	FA	E	MA	MA / FA	FA	E	E	MA	FA	FA											
		3.46	3.78	3.63		3.66	3.86	3.14	2.60		3.90	3.76	3.64											
6844	11/ 7	MA	MA	FA	MA	MA	MA	FA	FA	FA	E	FA / E	MA	MA	E	FA								
		3.29	3.37	3.29	3.10	3.07	3.25	2.88	3.16	3.27		3.57		3.80	3.35		3.47							
6845	11/ 6	MA	E	MA	FA	E	MA	MA	FA	MA	MA / MA	FA	FA	FA	FA	MA								
		3.32		3.83	2.96		3.61	3.71	3.43	3.55	3.61	3.36	3.04	3.42	3.47	3.36	3.78							
6846	12/ 8	MA	FA	MA	MA	MA	MA	FA	FA	FA / FA	FA	FA	FA	FA	FA	MA	MA							
		3.19	3.43	3.56	3.60	4.17	3.83	3.66	3.66	3.40	3.13	3.66	3.66	3.31	3.61	3.85	3.86							
6847		NOT PREGNANT																						
6848	6/12	MA	MA	FA	FA	FA	FA / MA	MA	MA	MA	MA	FA	MA	FA	FA	MA	MA							
		3.38	3.47	2.82	3.01	3.18	3.19	3.15	3.04	2.87	2.41	3.11	3.39	2.79	2.77	2.40	2.95							
6849	11/ 9	E	FA	MA	FA	E	FA	MA	FA	FA	MA / FA	FA	FA	FA	MA	MA	FA							
			3.27	3.51	3.07		3.17	3.46	3.23	3.26	3.48	3.41	3.61	3.07	3.57	3.44	3.43	3.48						
6850	7/ 8	MA	FA	FA	FA	MA	FA	MA / MA	MA	FA	FA	FA	FA	FA	FA									
		3.31	3.65	3.47	3.69	3.61	3.30	3.70	3.43	3.77	3.34	3.46	3.41	3.39	3.58									

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PROTOCOL 720-002: DEVELOPMENTAL TOXICITY STUDY OF HYAMINE® 1622 IN RATS

TABLE 19 (PAGE 5): FETAL SEX, VITAL STATUS AND BODY WEIGHT - INDIVIDUAL DATA

DOSAGE GROUP III			30 MG/KG/DAY																						
FETUS #			1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23
RAT #	CLs																								
6851		NOT PREGNANT																							
6852	9/ 9		E	E	E	MA	MA	FA	FA	MA	FA / MA	MA	FA	FA	MA	MA	MA	FA	MA						
						3.46	3.41	3.30	3.20	3.65	3.44	3.67	3.51	3.30	2.82	3.74	3.59	3.35	3.26	3.29					
6853	7/ 9		MA	MA	MA	FA	FA	MA	FA / MA	MA	MA	MA	FA	FA	FA	MA	FA	FA	MA	FA	FA				
			3.58	3.76	3.59	3.42	3.30	3.65	3.38	3.08	3.38	3.66	3.13	2.95	3.53	3.70	2.95	3.36							
6854	9/ 6		MA	FA	MA	FA	MA	MA	MA	FA	FA / FA	FA	FA	MA	MA										
			3.12	3.10	3.26	3.04	3.18	3.01	2.96	2.68	2.97	2.93	2.92	2.83	3.51	3.32									
6855	8/ 7		FA	MA	MA	FA	MA	FA / MA	MA	FA	MA	FA	MA	FA	MA										
			2.70	3.15	3.35	3.40	3.70	3.01	3.09	2.36	3.33	3.48	3.38	3.51	3.65										
6856	11/ 8		FA	FA	FA	FA	FA	FA	MA	MA	MA / FA	MA	FA	MA	FA	MA	MA								
			3.26	2.99	2.68	3.48	3.20	3.28	3.57	3.20	3.50	3.24	3.59	3.50	3.35	3.09	3.57	3.33							
6857	5/ 9		FA	FA	MA	MA	FA / MA	E	E	FA	MA	MA	E	FA	MA										
			3.08	3.15	3.62	3.41	2.98	3.49			2.97	3.23	3.17		3.36	3.06									
6858	12/ 6		MA	FA	FA	MA	MA	MA	MA	E	FA	MA	FA / FA	FA	FA	MA	FA	FA							
			3.13	3.14	3.15	3.27	3.14	3.37	3.34		3.23	3.36	3.40	3.04	2.65	3.40	3.33	3.17							
6859	10/ 7		MA	MA	FA	MA	FA	MA	MA	FA	E	MA / E	MA	FA	FA	FA	FA	MA							
			3.44	3.85	3.48	3.48	3.59	3.54	3.27	3.26		3.51		3.63	3.69	3.27	3.27	3.63							
6860	12/ 7		FA	FA	FA	MA	FA	FA	FA	FA	MA / MA	FA	FA	MA	MA	MA	FA								
			3.82	4.04	3.60	3.89	4.13	3.84	3.57	3.94	4.26	3.67	3.74	3.92	4.05	4.25	4.46	4.12							
6861	5/10		MA	FA	FA	MA / FA	MA	MA	MA	MA	MA	E	MA	MA	FA	FA									
			3.63	3.26	3.23	3.27	3.01	3.56	3.33	3.28	3.04		3.15	3.22	3.05	2.80									
6862	9/ 9		FA	MA	MA	MA	FA	FA	MA	FA / E	FA	FA	MA	MA	FA	FA									
			3.40	3.59	3.73	3.59	3.50	3.57	3.86	3.45		3.41	3.25	3.65	3.42	3.37	3.44								
6863	11/ 5		FA	MA	FA	FA	FA	FA	MA	FA	MA	MA / MA	FA	MA	MA	FA	MA	FA							
			2.77	3.17	2.82	3.07	2.91	3.08	2.91	2.54	3.06	3.21	3.18	3.16	2.37	3.28	3.14	3.06							

M = MALE F = FEMALE A = ALIVE D = DEAD E = EARLY RESORPTION L = LATE RESORPTION "/" DENOTES POSITION OF CERVIX
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PROTOCOL 720-002: DEVELOPMENTAL TOXICITY STUDY OF HYAMINE® 1622 IN RATS

TABLE 19 (PAGE 6): FETAL SEX, VITAL STATUS AND BODY WEIGHT - INDIVIDUAL DATA

DOSAGE GROUP III		30 MG/KG/DAY																						
FETUS #		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23
RAT #	CLs																							
6864	8/ 9	MA	FA	MA	FA	FA	MA	FA	FA / MA	FA	FA	FA	MA	MA	FA	MA								
		3.12	2.81	3.25	2.90	2.67	3.47	2.60	3.10	3.24	3.17	2.98	3.14	3.66	3.43	2.83	3.29							
6865	9/ 9	FA	FA	MA	MA	MA	MA	FA / FA	MA	MA	FA	MA	FA	MA										
		3.16	3.47	3.58	3.29	3.63	3.74	3.59	3.57	3.50	3.76	3.33	3.68	3.59	3.84									
6866	12/ 7	MA	MA	MA	FA	MA	FA	FA	FA	MA	FA / E	MA	MA	MA	MA	FA								
		3.69	3.88	3.65	3.80	3.45	3.17	3.04	3.38	2.99	3.01			3.86	3.78	3.06	3.52	3.68						
6867	10/ 6	MA	FA	MA	FA	MA	MA	MA	MA	MA / FA	FA	MA	FA	MA										
		3.11	3.29	3.24	2.44	3.31	3.55	3.16	3.51	3.45	3.15	3.03	3.63	3.17	3.41									
6868	10/10	MA	MA	MA	FA	FA / MA	MA	MA	MA	FA	MA	MA												
		3.67	3.81	3.99	3.16	3.22	3.73	3.94	3.97	3.88	3.52	3.57	3.52											
6869	7/ 9	FA	FA	MA	FA	MA	FA / MA	MA	FA	FA	MA	FA	MA	FA	MA									
		2.51	2.93	3.48	2.96	3.42	3.44	3.16	2.59	3.05	3.02	3.32	2.96	2.99	3.02	3.29								
6870	8/ 3	E	FA	FA	FA	MA	FA	MA	MA / MA	MA	MA													
		2.99	3.40	2.87	3.08	3.09	3.07	3.35	3.07	3.56	3.21													
6871	10/ 8	MA	MA	FA	FA	FA	MA	FA	E / FA	MA	FA	FA	MA	MA	MA	FA								
		3.36	3.62	3.26	3.40	3.30	3.70	3.19			3.23	3.49	2.98	3.27	3.53	3.60	3.36	3.48						
6872	6/ 9	MA	FA	MA	MA	MA	MA / MA	FA	MA	FA	E	FA	FA	FA										
		3.20	3.08	3.66	3.53	3.70	3.71	2.71	3.06	3.68	2.68			3.15	3.19	3.33								
6873	7/ 8	MA	FA	MA	FA	MA	FA	FA / FA	MA	MA	MA	MA	MA	FA	FA	MA								
		3.28	3.34	3.43	3.06	3.30	3.14	2.73	2.90	3.23	3.19	3.43	3.56	3.21	3.32	3.50								
6874	5/11	MA	FA	FA	FA	MA / FA	MA	MA	MA	E	MA	FA	FA	MA	MA	FA								
		3.23	2.93	3.11	2.95	3.10	2.10	2.87	3.32	2.92			3.00	3.21	2.64	3.20	3.22	3.10						
6875	8/10	MA	E	MA	FA	FA	MA	FA / MA	MA	MA	MA	FA	MA	MA	MA	MA								
		3.42			3.40	3.48	3.52	3.46	3.62	3.24	3.24	3.45	3.39	3.27	3.24	3.29	3.40	3.21						

M = MALE F = FEMALE A = ALIVE D = DEAD E = EARLY RESORPTION L = LATE RESORPTION "/" DENOTES POSITION OF CERVIX
CLs = CORPORA LUTEA/OVARY FETAL BODY WEIGHTS WERE RECORDED IN GRAMS (G).

PROTOCOL 720-002: DEVELOPMENTAL TOXICITY STUDY OF HYAMINE® 1622 IN RATS

TABLE 19 (PAGE 7): FETAL SEX, VITAL STATUS AND BODY WEIGHT - INDIVIDUAL DATA

DOSAGE GROUP IV			100 MG/KG/DAY																						
FETUS #			1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23
RAT #	CLs																								
6876	9/ 8	FA	MA	FA	MA	FA	FA	MA	FA / MA	FA	E	FA	FA	MA	MA	MA									
		2.96	3.52	3.27	3.48	3.06	3.19	3.49	2.44	3.32	3.42		3.07	3.27	3.42	2.93	3.29								
6877	6/ 9	E	FA	FA	MA	MA / MA	FA	MA	FA	FA	FA	FA	FA	FA	FA										
			3.38	3.25	3.55	3.36	3.33	3.09	3.63	3.14	3.24	3.31	3.15	3.24	3.63										
6878	12/ 4	FA	FA	FA	MA	MA	FA	MA	MA	MA	FA	MA / MA	MA	FA											
		2.98	3.43	3.28	4.14	4.05	4.05	4.14	3.70	3.29	3.59	4.21	4.03	3.89	3.56										
6879	8/10	MA	E	FA	FA	E	FA	MA	MA / MA	E	FA	FA	MA	FA	MA	FA	E								
		2.64		2.71	2.87		2.86	3.27	3.28	3.18		2.51	3.01	2.93	2.98	3.26	3.02								
6880	7/ 9	MA	MA	FA	MA	FA	FA / FA	MA	MA	MA	MA	MA	FA	MA											
		3.53	3.77	3.34	3.63	3.19	3.28	2.97	3.35	3.00	3.27	2.67	3.36	3.26	3.53										
6881		NOT PREGNANT																							
6882	7/ 8	FA	MA	MA	MA	MA	MA / FA	MA	E	FA	E	FA	FA												
		3.12	3.40	3.44	3.24	3.25	3.13	3.37	3.55		2.99		3.16	3.40											
6883	8/ 8	FA	FA	FA	FA	FA	MA	FA / MA	MA	FA	FA	FA	MA												
		3.16	3.05	3.45	3.54	3.47	3.30	3.41	3.25	3.92	3.23	3.17	3.17	2.90	3.32										
6884	7/10	FA	E	MA	FA	MA	E	FA / MA	FA	FA	MA	MA	MA	MA	MA	MA									
		2.97		3.48	3.15	3.38		3.24	3.68	2.80	3.36	3.60	3.61	3.33	3.55	3.67	3.54								
6885	6/ 9	E	E	FA	FA	E / MA	FA	MA	E	FA	FA	FA	FA												
				3.43	3.82		1.95	3.49	3.72		3.71	3.55	3.27	3.49											
6886	9/ 8	FA	FA	MA	FA	MA	MA	FA	MA	MA / E	E	FA	FA	MA	FA	MA									
		2.95	3.29	2.87	3.12	3.10	3.31	3.25	3.01	2.89		3.16	3.51	3.06	3.19	3.31									
6887	8/ 8	MA	FA	E	MA	FA	FA / MA	MA	FA	FA	FA	MA	MA												
		3.74	3.50		3.70	3.59	3.91	3.69	2.88	3.78	3.60	3.47	3.43	3.58											
6888	11/ 9	FA	MA	MA	MA	FA	MA	MA	FA	FA / FA	MA	MA	MA	FA	MA	FA	MA								
		2.81	3.31	3.23	4.41	2.62	2.90	2.88	2.09	3.05	2.75	3.25	2.94	3.23	2.73	3.05	2.55	2.75							

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PROTOCOL 720-002: DEVELOPMENTAL TOXICITY STUDY OF HYAMINE® 1622 IN RATS

TABLE 19 (PAGE 8): FETAL SEX, VITAL STATUS AND BODY WEIGHT - INDIVIDUAL DATA

DOSAGE GROUP IV		100 MG/KG/DAY																						
FETUS #		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23
RAT #	CLs																							
6889	9/ 8	MA	MA	MA	FA	MA	MA	MA	MA	MA / FA	FA	MA	FA	MA	MA	MA								
		3.07	3.04	2.89	2.75	3.31	3.27	3.05	3.16	3.34	2.76	2.78	3.30	2.94	3.24	3.43	3.54							
6890	7/ 8	FA	MA	FA	MA	MA	MA / MA	MA	FA	FA	FA	MA	MA											
		3.12	3.65	3.76	3.51	3.54	3.94	3.35	3.57	3.42	3.20	3.24	3.85	3.59										
6891	9/ 7	FA	MA	MA	MA	MA	FA	MA	FA	FA / E	MA	FA	FA	L	FA	MA								
		2.68	2.89	3.17	3.04	3.31	3.14	3.04	2.86	3.44		3.02	2.97	2.72		2.94	3.03							
6892	8/12	FA	MA	FA	MA	E	MA / FA	MA	FA	MA	FA	FA	MA	FA	FA	FA	MA							
		3.53	3.53	3.10	3.57		3.86	3.16	2.91	3.17	3.18	3.66	3.24	3.53	3.61	3.06	3.35	3.96						
6893	9/ 6	E	MA	MA	MA	MA	MA	MA	MA / FA	FA	FA	FA	FA	FA	MA									
			3.02	2.84	2.93	3.04	2.88	3.27	3.29	3.01	2.96	2.82	3.36	3.03	3.13									
6894	8/ 8	E	MA	MA	MA	E	FA	FA	MA / FA	FA	FA	FA	FA	FA	FA	FA	FA							
			3.66	3.69	3.38		3.08	2.42	3.66	3.32	3.08	3.19	3.12	3.61	3.28	3.72	3.23							
6895	12/ 5	L	E	MA	MA	FA	MA	FA	FA	MA	FA	FA	FA / FA	MA	FA	FA	MA							
				2.82	2.78	3.23	3.35	3.03	2.99	3.26	3.06	2.80	3.34	3.14	3.12	3.26	2.96	3.14						
6896	9/ 9	MA	MA	FA	MA	FA	MA	MA / MA	MA	MA	MA	E	FA	FA	FA	E								
		3.30	3.69	3.16	3.33	3.38	3.52	3.15	3.08	3.34	3.39	3.52		3.20	3.32	3.06								
6897	6/12	MA	MA	MA	FA	E	FA / E	FA	E	FA	FA	FA	FA	FA	MA	FA	MA	FA						
		2.44	2.58	3.04	2.53		2.64		1.68		2.37	2.36	2.22	2.39	2.30	2.74	2.73	2.91	2.33					
6898	9/11	MA	MA	FA	FA	FA	FA	MA / MA	MA	MA	MA	FA	MA	MA	MA	MA	FA							
		3.67	3.93	3.51	3.55	3.71	3.50	4.04	3.62	3.64	3.82	3.49	3.42	3.61	3.80	3.85	3.78							
6899	9/ 6	MA	E	FA	FA	FA	FA	FA	MA	FA / MA	FA	FA	FA	MA	MA									
		2.78		3.31	3.23	2.12	3.40	3.28	3.38	3.40	3.05	3.55	3.02	2.87	3.19	3.42								
6900	13/ 5	MA	MA	MA	FA	FA	MA	MA	MA	FA	MA	FA / E	FA	FA	FA	FA								
		2.88	3.01	3.05	2.87	2.90	2.85	2.99	3.27	2.39	2.96	2.90		3.03	3.41	2.84	2.98							

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PROTOCOL 720-002: DEVELOPMENTAL TOXICITY STUDY OF HYAMINE® 1622 IN RATS

TABLE 19 (PAGE 9): FETAL SEX, VITAL STATUS AND BODY WEIGHT - INDIVIDUAL DATA

DOSAGE GROUP V				170 MG/KG/DAY																				
FETUS #		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23
RAT #	CLS																							
6901	9/ 4	FA	MA	FA	MA	MA	MA	FA	FA	FA / MA	MA	MA												
		2.43	3.11	2.39	3.03	2.88	2.96	2.78	2.74	2.49	3.01	2.96	2.89											
6902	10/ 7	MA	FA	FA	FA	FA	FA	MA	MA	FA / FA	MA	MA	MA	MA										
		4.06	3.79	3.48	3.53	3.58	3.84	3.55	3.67	3.64	3.33	3.82	3.54	3.86	3.62									
6903	10/ 8	MA	FA	MA	FA	FA	MA	FA	FA	MA	MA / MA	FA	MA	MA	FA	FA	FA	MA						
		3.80	3.50	3.94	3.73	4.00	3.79	3.75	3.71	3.90	3.55	4.09	3.75	3.90	3.93	3.66	3.60	3.21	3.94					
6904	12/ 7	FA	MA	MA	MA	FA	MA	FA	FA	MA / FA	FA	FA	MA	FA	MA									
		3.49	3.59	3.11	3.33	3.58	3.59	3.38	2.75	3.09	3.59	3.60	3.31	3.49	3.27	3.82								
6905	11/ 9	FA	FA	FA	FA	FA	MA	FA	FA	FA / MA	FA	FA	FA	FA	MA	MA	FA	FA						
		3.17	3.80	3.23	3.90	3.72	4.01	3.80	3.51	3.65	3.84	3.84	3.64	3.60	3.79	3.43	3.47	3.81						
6906	7/10	FA	FA	MA	FA	FA	MA	FA / MA	MA	MA	FA	MA	E	FA	MA	MA	E							
		3.20	3.55	3.94	3.36	3.45	3.37	3.84	3.89	3.73	3.45	3.70				3.63	3.73	3.79						
6907	8/ 7	FA	E	E	E	MA	MA	MA / E	E	E	E	E	MA	E	E									
		3.63				3.88	3.61	3.76					3.93											
6908	11/ 8	FA	MA	MA	MA	FA	FA / MA	MA	MA	FA	MA	E	E											
		3.63	3.24	3.36	3.37	3.18	3.18	2.48	3.25	3.62	3.15	3.40												
6909	7/ 9	MA	E	FA	MA	MA	MA / FA	MA	FA	MA	FA	FA	FA											
		3.28			3.52	3.65	3.16	3.63	3.11	3.35	3.28	3.67	3.33	3.38	3.68									
6910		FOUND DEAD ON DAY 11 OF GESTATION																						
6911	9/ 8	E	MA	MA	E	MA	MA	FA	FA / MA	MA	MA	FA	FA	FA	FA									
		3.55	3.50			3.46	3.61	3.08	3.35	3.67	3.22	3.68	2.93	3.37	3.31	3.59								
6912	11/ 4	FA	MA	FA	MA	MA	MA	FA	MA	MA	MA /													
		3.44	3.28	3.26	3.21	3.23	3.60	3.23	3.56	3.51	3.63													
6913	9/ 6	FA	FA	MA	MA	FA	FA	MA	MA	FA / MA	MA	MA	FA	MA										
		2.64	2.61	2.71	2.99	2.51	2.65	2.89	2.77	2.59	2.63	2.84	2.92	2.81	2.70									

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PROTOCOL 720-002: DEVELOPMENTAL TOXICITY STUDY OF HYAMINE® 1622 IN RATS

TABLE 19 (PAGE 10): FETAL SEX, VITAL STATUS AND BODY WEIGHT - INDIVIDUAL DATA

DOSAGE GROUP V		170 MG/KG/DAY																						
FETUS #		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23
RAT #	CLs																							
6914	6/12	FA	MA	MA	FA	MA / FA	MA	FA	E	FA	E	FA	FA	FA	MA	MA								
		3.23	3.55	3.48	3.21	3.89	3.33	3.80	2.45		3.55		3.50	3.59	3.14	3.42	3.74							
6915	7/ 9	MA	FA	MA	E	FA	MA	MA / MA	MA	FA	FA	FA	MA	MA	MA	FA								
		2.06	3.45	3.23		3.29	2.89	3.34	3.55	2.18	3.15	1.82	2.54	3.26	2.48	3.27	3.13							
6916	9/ 7	MA	MA	MA	FA	MA	FA	MA	E	MA / FA	FA	MA	FA	MA										
		3.67	3.76	3.96	3.62	3.76	3.58	3.68		3.51	3.65	3.53	3.58	3.80	3.42									
6917	9/ 8	E	FA	FA	MA	FA	E	MA	MA / MA	FA														
			2.41	2.72	2.42	2.05		2.36	2.70	2.61	2.19													
6918		FOUND DEAD ON DAY 13 OF GESTATION																						
6919	2/ 5	MA / MA	FA	FA	FA	MA																		
		3.51	3.10	2.26	3.52	3.15	2.36																	
6920	9/ 7	FA	FA	FA	FA	MA	MA	FA	FA / FA	FA	MA	MA	MA	MA										
		3.19	4.38	3.12	2.85	3.50	3.50	3.10	2.96	3.21	3.64	3.65	3.34	2.58	3.37									
6921		FOUND DEAD ON DAY 16 OF GESTATION																						
6922	10/ 9	MA	FA	MA	FA	MA	MA	MA	MA / MA	FA	FA	FA	FA	FA										
		2.99	2.99	3.38	3.09	3.01	2.93	2.95	3.09	3.17	3.16	3.35	2.96	3.04	2.87									
6923		FOUND DEAD ON DAY 12 OF GESTATION																						
6924	11/ 9	E	MA	FA	FA	FA	E	FA	FA	MA	FA	FA / MA	MA	MA	FA									
			2.81	2.69	3.07	3.13		2.80	3.07	3.01	2.89	2.53	2.85	3.20	3.10	2.81								
6925	7/12	FA	MA	MA	FA	MA	MA	FA / MA	MA	FA	MA	MA	FA	MA	FA	FA	FA	MA						
		2.05	3.49	3.01	2.88	3.50	3.34	3.07	3.39	3.07	2.90	3.20	3.40	2.91	3.13	2.80	2.97	3.03	3.35					
M = MALE F = FEMALE A = ALIVE D = DEAD E = EARLY RESORPTION L = LATE RESORPTION "/" DENOTES POSITION OF CERVIX																								
CLs = CORPORA LUTEA/OVARY FETAL BODY WEIGHTS WERE RECORDED IN GRAMS (G).																								

PROTOCOL 720-002: DEVELOPMENTAL TOXICITY STUDY OF HYAMINE® 1622 IN RATS

TABLE 20 (PAGE 1): FETAL ALTERATIONS - INDIVIDUAL DATA

DOSAGE GROUP I		0(VEHICLE) MG/KG/DAY					
RAT NUMBER	SPECIMENS WITH ANY ALTERATIONS N(%)	GROSS EXTERNAL EXAMINATION		SOFT TISSUE EXAMINATION		SKELETAL EXAMINATION	
		N/N	DESCRIPTION	N/N	DESCRIPTION	N/N	DESCRIPTION
6801	1(7.1)	1/14	FETUS 1 HEAD: EXENCEPHALY	0/ 7		1/ 7	FETUS 1 SKULL: MAXILLAE AND PREMAXILLAE, SHORT; NASALS, SHORT; BASISPHENOID, INCOMPLETELY OSSIFIED; FRONTALS, NOT OSSIFIED; PARIETALS, NOT OSSIFIED THORACIC VERTEBRAE: CENTRUM, BIFID, 11th STERNAL CENTRA: 1ST, NOT OSSIFIED
6802	0(0.0)	0/13		0/ 6		0/ 7	
6803	0(0.0)	0/12		0/ 6		0/ 6	
6804	0(0.0)	0/15		0/ 7		0/ 8	

N/N = NUMBER OF SPECIMENS WITH ALTERATIONS/NUMBER OF SPECIMENS EXAMINED

PROTOCOL 720-002: DEVELOPMENTAL TOXICITY STUDY OF HYAMINE® 1622 IN RATS

TABLE 20 (PAGE 2): FETAL ALTERATIONS - INDIVIDUAL DATA

DOSAGE GROUP 1		0 (VEHICLE) MG/KG/DAY					
RAT NUMBER	SPECIMENS WITH ANY ALTERATIONS N (%)	GROSS EXTERNAL EXAMINATION		SOFT TISSUE EXAMINATION		SKELETAL EXAMINATION	
		N/N	DESCRIPTION	N/N	DESCRIPTION	N/N	DESCRIPTION
6805	2 (15.4)	0/13		0/ 6		2/ 7	FETUS 5 LUMBAR VERTEBRAE: ARCH, INCOMPLETELY OSSIFIED, bilateral 6th RIBS: WAVY, right 4th - 13th, left 4th - 7th, 11th and 12th; INCOMPLETELY OSSIFIED (HYPOPLASTIC), right 10th and 11th, left 10th and 12th PELVIS: PUBIS, INCOMPLETELY OSSIFIED, left FETUS 9 RIBS: INCOMPLETELY OSSIFIED (HYPOPLASTIC), right 9th PELVIS: ISCHIUM, INCOMPLETELY OSSIFIED, bilateral; PUBIS, INCOMPLETELY OSSIFIED, bilateral
6806	0 (0.0)	0/16		0/ 8		0/ 8	
6807	1 (7.1)	0/14		0/ 7		1/ 7	FETUS 5 CERVICAL VERTEBRAE: CERVICAL RIB AT 7TH CERVICAL VERTEBRA, right

N/N = NUMBER OF SPECIMENS WITH ALTERATIONS/NUMBER OF SPECIMENS EXAMINED

PROTOCOL 720-002: DEVELOPMENTAL TOXICITY STUDY OF HYAMINE® 1622 IN RATS

TABLE 20 (PAGE 3): FETAL ALTERATIONS - INDIVIDUAL DATA

DOSAGE GROUP I		0 (VEHICLE) MG/KG/DAY					
RAT NUMBER	SPECIMENS WITH ANY ALTERATIONS N (%)	GROSS EXTERNAL EXAMINATION		SOFT TISSUE EXAMINATION		SKELETAL EXAMINATION	
		N/N	DESCRIPTION	N/N	DESCRIPTION	N/N	DESCRIPTION
6808	2 (13.3)	0/15		0/ 7		2/ 8	FETUS 9 CERVICAL VERTEBRAE: CERVICAL RIB AT 7TH CERVICAL VERTEBRA, right FETUS 13 THORACIC VERTEBRAE: CENTRUM, BIFID, 11th
6809	0 (0.0)	0/10		0/ 5		0/ 5	
6810	4 (25.0)	0/16		0/ 8		4/ 8	FETUS 3 RIBS: WAVY, right 7th, 9th - 12th; INCOMPLETELY OSSIFIED (HYPOPLASTIC), right 7th, 9th - 12th FETUS 5 LUMBAR VERTEBRAE: ARCH, INCOMPLETELY OSSIFIED, right 6th FETUS 7 RIBS: WAVY, right 6th, 10th and 11th

N/N = NUMBER OF SPECIMENS WITH ALTERATIONS/NUMBER OF SPECIMENS EXAMINED

PROTOCOL 720-002: DEVELOPMENTAL TOXICITY STUDY OF HYAMINE® 1622 IN RATS

TABLE 20 (PAGE 4): FETAL ALTERATIONS - INDIVIDUAL DATA

DOSAGE GROUP I		0(VEHICLE) MG/KG/DAY					
RAT NUMBER	SPECIMENS WITH ANY ALTERATIONS N(%)	GROSS EXTERNAL EXAMINATION		SOFT TISSUE EXAMINATION		SKELETAL EXAMINATION	
		N/N	DESCRIPTION	N/N	DESCRIPTION	N/N	DESCRIPTION
6810 (cont.)							FETUS 11 RIBS: WAVY, right 9th - 11th, left 11th and 12th; INCOMPLETELY OSSIFIED (HYPOPLASTIC), right 10th and 11th, left 11th and 12th
6811	0(0.0)	0/13		0/ 6		0/ 7	
6812	0(0.0)	0/12		0/ 6		0/ 6	
6813	0(0.0)	0/16		0/ 8		0/ 8	
6814	0(0.0)	0/14		0/ 7		0/ 7	
6815	5(29.4)	1/17	FETUS 18 PALATE: CLEFT, medial JAW: MICROGNATHIA	1/ 8	FETUS 18 PALATE: CLEFT, medial	4/ 9	FETUS 1 PELVIS: PUBIS, INCOMPLETELY OSSIFIED, bilateral FETUS 8 PELVIS: PUBIS, INCOMPLETELY OSSIFIED, bilateral

N/N = NUMBER OF SPECIMENS WITH ALTERATIONS/NUMBER OF SPECIMENS EXAMINED

PROTOCOL 720-002: DEVELOPMENTAL TOXICITY STUDY OF HYAMINE® 1622 IN RATS

TABLE 20 (PAGE 5): FETAL ALTERATIONS - INDIVIDUAL DATA

DOSAGE GROUP I		0(VEHICLE) MG/KG/DAY					
RAT NUMBER	SPECIMENS WITH ANY ALTERATIONS N(%)	GROSS EXTERNAL EXAMINATION		SOFT TISSUE EXAMINATION		SKELETAL EXAMINATION	
		N/N	DESCRIPTION	N/N	DESCRIPTION	N/N	DESCRIPTION
6815 (cont.)							FETUS 12 PELVIS: ISCHIUM, INCOMPLETELY OSSIFIED, bilateral; PUBIS, INCOMPLETELY OSSIFIED, bilateral
							FETUS 19 PELVIS: PUBIS, INCOMPLETELY OSSIFIED, bilateral
6816	0(0.0)	0/14		0/ 7		0/ 7	
6817	1(7.1)	0/14		0/ 7 ^a		1/ 7	FETUS 7 PELVIS: PUBIS, INCOMPLETELY OSSIFIED, left
6818	1(7.1)	0/14		0/ 7		1/ 7	FETUS 3 RIBS: WAVY, bilateral 4th - 12th PELVIS: ISCHIUM, INCOMPLETELY OSSIFIED, right; PUBIS, INCOMPLETELY OSSIFIED, bilateral

N/N = NUMBER OF SPECIMENS WITH ALTERATIONS/NUMBER OF SPECIMENS EXAMINED

a. The head of fetus 6817-10 was examined at soft tissue evaluation; all other observations were not recorded.

PROTOCOL 720-002: DEVELOPMENTAL TOXICITY STUDY OF HYAMINE® 1622 IN RATS

TABLE 20 (PAGE 6): FETAL ALTERATIONS - INDIVIDUAL DATA

DOSAGE GROUP I		0(VEHICLE) MG/KG/DAY					
RAT NUMBER	SPECIMENS WITH ANY ALTERATIONS N(%)	GROSS EXTERNAL EXAMINATION		SOFT TISSUE EXAMINATION		SKELETAL EXAMINATION	
		N/N	DESCRIPTION	N/N	DESCRIPTION	N/N	DESCRIPTION
6819	2(15.4)	0/13		0/ 6		2/ 7	FETUS 1 RIBS: WAVY, right 5th - 12th, left 7th, 10th and 11th STERNAL CENTRA: 1ST, INCOMPLETELY OSSIFIED PELVIS: PUBIS, INCOMPLETELY OSSIFIED, bilateral FETUS 7 PELVIS: PUBIS, INCOMPLETELY OSSIFIED, bilateral
6820	0(0.0)	0/15		0/ 7		0/ 8	
6821	0(0.0)	0/18		0/ 9		0/ 9	
6822	0(0.0)	0/16		0/ 8		0/ 8	
6823	0(0.0)	0/16		0/ 8		0/ 8	
6824	1(7.7)	0/13		1/ 6	FETUS 4 VESSELS: INOMINATE, ABSENT	0/ 7	
6825	1(6.2)	0/16		0/ 8		1/ 8	FETUS 5 THORACIC VERTEBRAE: CENTRUM, BIFID, 10th and 11th

N/N = NUMBER OF SPECIMENS WITH ALTERATIONS/NUMBER OF SPECIMENS EXAMINED

PROTOCOL 720-002: DEVELOPMENTAL TOXICITY STUDY OF HYAMINE® 1622 IN RATS

TABLE 20 (PAGE 7): FETAL ALTERATIONS - INDIVIDUAL DATA

DOSAGE GROUP II		10 MG/KG/DAY					
RAT NUMBER	SPECIMENS WITH ANY ALTERATIONS N (%)	GROSS EXTERNAL EXAMINATION		SOFT TISSUE EXAMINATION		SKELETAL EXAMINATION	
		N/N	DESCRIPTION	N/N	DESCRIPTION	N/N	DESCRIPTION
3100	0(0.0)	0/17		0/ 8		0/ 9	
6827	0(0.0)	0/13		0/ 6		0/ 7	
6828	0(0.0)	0/15		0/ 7		0/ 8	
6829	1(7.7)	0/13		0/ 6		1/ 7	FETUS 13 PELVIS: ISCHIUM, INCOMPLETELY OSSIFIED, left; PUBIS, INCOMPLETELY OSSIFIED, bilateral
6830	0(0.0)	0/15	FETUS 9 (DEAD FETUS) APPEARED NORMAL FOR DEVELOPMENTAL AGE	0/ 7		0/ 8	
6831	1(6.2)	0/16		0/ 8		1/ 8	FETUS 3 RIBS: WAVY, bilateral 11th; INCOMPLETELY OSSIFIED (HYPOPLASTIC), bilateral 11th
6832	0(0.0)	0/15		0/ 7		0/ 8	
6833	0(0.0)	0/13		0/ 6		0/ 7	

N/N = NUMBER OF SPECIMENS WITH ALTERATIONS/NUMBER OF SPECIMENS EXAMINED

PROTOCOL 720-002: DEVELOPMENTAL TOXICITY STUDY OF HYAMINE® 1622 IN RATS

TABLE 20 (PAGE 8): FETAL ALTERATIONS - INDIVIDUAL DATA

DOSAGE GROUP II		10 MG/KG/DAY					
RAT NUMBER	SPECIMENS WITH ANY ALTERATIONS N(%)	GROSS EXTERNAL EXAMINATION		SOFT TISSUE EXAMINATION		SKELETAL EXAMINATION	
		N/N	DESCRIPTION	N/N	DESCRIPTION	N/N	DESCRIPTION
6834	6 (46.2)	0/13		0/ 6		6/ 7	<p>FETUS 1</p> <p>LUMBAR VERTEBRAE: ARCH, INCOMPLETELY OSSIFIED, left 6th</p> <p>RIBS: WAVY, right 5th - 13th, left 4th - 13th; INCOMPLETELY OSSIFIED (HYPOPLASTIC), bilateral 6th - 13th</p> <p>PELVIS: ISCHIUM, INCOMPLETELY OSSIFIED, bilateral; PUBIS, INCOMPLETELY OSSIFIED, left</p> <p>FETUS 3</p> <p>LUMBAR VERTEBRAE: ARCH, INCOMPLETELY OSSIFIED, right 5th, left 6th</p> <p>RIBS: INCOMPLETELY OSSIFIED (HYPOPLASTIC), right 13th</p> <p>PELVIS: ISCHIUM, INCOMPLETELY OSSIFIED, left</p> <p>FETUS 7</p> <p>STERNAL CENTRA: 1ST, INCOMPLETELY OSSIFIED</p>

N/N = NUMBER OF SPECIMENS WITH ALTERATIONS/NUMBER OF SPECIMENS EXAMINED

PROTOCOL 720-002: DEVELOPMENTAL TOXICITY STUDY OF HYAMINE® 1622 IN RATS

TABLE 20 (PAGE 9): FETAL ALTERATIONS - INDIVIDUAL DATA

DOSAGE GROUP II		10 MG/KG/DAY					
RAT NUMBER	SPECIMENS WITH ANY ALTERATIONS N(%)	GROSS EXTERNAL EXAMINATION		SOFT TISSUE EXAMINATION		SKELETAL EXAMINATION	
		N/N	DESCRIPTION	N/N	DESCRIPTION	N/N	DESCRIPTION
6834 (cont.)							FETUS 9 RIBS: WAVY, right 8th - 11th, left 11th; INCOMPLETELY OSSIFIED (HYPOPLASTIC), right 8th, 10th and 11th STERNAL CENTRA: 1ST, INCOMPLETELY OSSIFIED; 2ND, INCOMPLETELY OSSIFIED PELVIS: ISCHIUM, INCOMPLETELY OSSIFIED, left FETUS 11 PELVIS: ISCHIUM, INCOMPLETELY OSSIFIED, right FETUS 13 LUMBAR VERTEBRAE: ARCH, INCOMPLETELY OSSIFIED, left 5th STERNAL CENTRA: 1ST, INCOMPLETELY OSSIFIED
6835	SACRIFICED ON DAY 20 OF GESTATION; DAM IN THE PROCESS OF DELIVERY						
N/N = NUMBER OF SPECIMENS WITH ALTERATIONS/NUMBER OF SPECIMENS EXAMINED							

PROTOCOL 720-002: DEVELOPMENTAL TOXICITY STUDY OF HYAMINE® 1622 IN RATS

TABLE 20 (PAGE 10): FETAL ALTERATIONS - INDIVIDUAL DATA

DOSAGE GROUP II		10 MG/KG/DAY					
RAT NUMBER	SPECIMENS WITH ANY ALTERATIONS N(%)	GROSS EXTERNAL EXAMINATION		SOFT TISSUE EXAMINATION		SKELETAL EXAMINATION	
		N/N	DESCRIPTION	N/N	DESCRIPTION	N/N	DESCRIPTION
6836	2(13.3)	0/15		0/ 7		2/ 8	FETUS 2 RIBS: WAVY, right 6th - 8th, 10th - 12th, left 11th and 12th FETUS 9 STERNAL CENTRA: 1ST, INCOMPLETELY OSSIFIED; 2ND, INCOMPLETELY OSSIFIED PELVIS: PUBIS, INCOMPLETELY OSSIFIED, bilateral
6837	2(15.4)	1/13	FETUS 6 EYES: BULGE DEPRESSED, left a	1/ 6	FETUS 6 EYES: MICROPHthalmia, left	1/ 7	FETUS 14 PELVIS: PUBIS, INCOMPLETELY OSSIFIED, right
6838	0(0.0)	0/14		0/ 7		0/ 7	
6839	0(0.0)	0/17		0/ 8		0/ 9	
6840	0(0.0)	0/10		0/ 5		0/ 5	
6841	0(0.0)	0/13		0/ 6		0/ 7	
6842	1(6.7)	0/15		0/ 7		1/ 8	FETUS 9 STERNAL CENTRA: 1ST, INCOMPLETELY OSSIFIED
6843	1(10.0)	0/10		0/ 5		1/ 5	FETUS 8 STERNAL CENTRA: 1ST, INCOMPLETELY OSSIFIED

N/N = NUMBER OF SPECIMENS WITH ALTERATIONS/NUMBER OF SPECIMENS EXAMINED

a. First observed at soft tissue examination.

PROTOCOL 720-002: DEVELOPMENTAL TOXICITY STUDY OF HYAMINE® 1622 IN RATS

TABLE 20 (PAGE 11): FETAL ALTERATIONS - INDIVIDUAL DATA

DOSAGE GROUP II		10 MG/KG/DAY					
RAT NUMBER	SPECIMENS WITH ANY ALTERATIONS N(%)	GROSS EXTERNAL EXAMINATION		SOFT TISSUE EXAMINATION		SKELETAL EXAMINATION	
		N/N	DESCRIPTION	N/N	DESCRIPTION	N/N	DESCRIPTION
6844	1(7.7)	0/13		0/ 6		1/ 7	FETUS 16 THORACIC VERTEBRAE: CENTRUM, BIFID, 12th
6845	0(0.0)	0/14		0/ 7		0/ 7	
6846	1(6.2)	0/16		0/ 8		1/ 8	FETUS 9 THORACIC VERTEBRAE: HEMIVERTEBRA, left 13th, arch with attached rib
6847	NOT PREGNANT						
6848	1(6.2)	0/16		1/ 8	FETUS 10 VESSELS: COMMON CAROTID ORIGINATES FROM THE INOMINATE	0/ 8	
6849	3(20.0)	0/15		0/ 7		3/ 8	FETUS 2 STERNAL CENTRA: 2ND, INCOMPLETELY OSSIFIED FETUS 9 STERNAL CENTRA: 1ST, INCOMPLETELY OSSIFIED FETUS 13 CERVICAL VERTEBRAE: CERVICAL RIB AT 7TH CERVICAL VERTEBRA, left STERNAL CENTRA: 1ST, INCOMPLETELY OSSIFIED
6850	0(0.0)	0/14		0/ 7		0/ 7	

N/N = NUMBER OF SPECIMENS WITH ALTERATIONS/NUMBER OF SPECIMENS EXAMINED

PROTOCOL 720-002: DEVELOPMENTAL TOXICITY STUDY OF HYAMINE® 1622 IN RATS

TABLE 20 (PAGE 12): FETAL ALTERATIONS - INDIVIDUAL DATA

DOSAGE GROUP III		30 MG/KG/DAY					
RAT NUMBER	SPECIMENS WITH ANY ALTERATIONS N(%)	GROSS EXTERNAL EXAMINATION		SOFT TISSUE EXAMINATION		SKELETAL EXAMINATION	
		N/N	DESCRIPTION	N/N	DESCRIPTION	N/N	DESCRIPTION
6851	NOT PREGNANT						
6852	0(0.0)	0/15		0/ 7		0/ 8	
6853	2(12.5)	0/16		0/ 8		2/ 8	FETUS 7 CERVICAL VERTEBRAE: CERVICAL RIB AT 7TH CERVICAL VERTEBRA, left FETUS 9 CERVICAL VERTEBRAE: CERVICAL RIB AT 7TH CERVICAL VERTEBRA, right
6854	0(0.0)	0/14		0/ 7		0/ 7	
6855	0(0.0)	0/13		0/ 6		0/ 7	
6856	0(0.0)	0/16		0/ 8		0/ 8	
6857	0(0.0)	0/11		0/ 5		0/ 6	
6858	1(6.7)	0/15		0/ 7		1/ 8	FETUS 3 PELVIS: PUBIS, INCOMPLETELY OSSIFIED, bilateral
6859	0(0.0)	0/14		0/ 7		0/ 7	

N/N = NUMBER OF SPECIMENS WITH ALTERATIONS/NUMBER OF SPECIMENS EXAMINED

PROTOCOL 720-002: DEVELOPMENTAL TOXICITY STUDY OF HYAMINE® 1622 IN RATS

TABLE 20 (PAGE 13): FETAL ALTERATIONS - INDIVIDUAL DATA

DOSAGE GROUP III		30 MG/KG/DAY					
RAT NUMBER	SPECIMENS WITH ANY ALTERATIONS N (%)	GROSS EXTERNAL EXAMINATION		SOFT TISSUE EXAMINATION		SKELETAL EXAMINATION	
		N/N	DESCRIPTION	N/N	DESCRIPTION	N/N	DESCRIPTION
6860	1(6.2)	0/16		0/ 8		1/ 8	FETUS 1 PELVIS: PUBIS, INCOMPLETELY OSSIFIED, right
6861	2(15.4)	0/13		0/ 6		2/ 7	FETUS 3 CERVICAL VERTEBRAE: CERVICAL RIB AT 7TH CERVICAL VERTEBRA, bilateral FETUS 14 CERVICAL VERTEBRAE: CERVICAL RIB AT 7TH CERVICAL VERTEBRA, right
6862	5(35.7)	0/14		0/ 7		5/ 7	FETUS 1 PELVIS: ISCHIUM, INCOMPLETELY OSSIFIED, bilateral FETUS 3 STERNAL CENTRA: 1ST, INCOMPLETELY OSSIFIED PELVIS: ISCHIUM, INCOMPLETELY OSSIFIED, right; PUBIS, INCOMPLETELY OSSIFIED, right

N/N = NUMBER OF SPECIMENS WITH ALTERATIONS/NUMBER OF SPECIMENS EXAMINED

PROTOCOL 720-002: DEVELOPMENTAL TOXICITY STUDY OF HYAMINE® 1622 IN RATS

TABLE 20 (PAGE 14): FETAL ALTERATIONS - INDIVIDUAL DATA

DOSAGE GROUP III		30 MG/KG/DAY					
RAT NUMBER	SPECIMENS WITH ANY ALTERATIONS N(%)	GROSS EXTERNAL EXAMINATION		SOFT TISSUE EXAMINATION		SKELETAL EXAMINATION	
		N/N	DESCRIPTION	N/N	DESCRIPTION	N/N	DESCRIPTION
6862 (cont.)							FETUS 5 LUMBAR VERTEBRAE: ARCH, INCOMPLETELY OSSIFIED, left 6th STERNAL CENTRA: 1ST, INCOMPLETELY OSSIFIED; 2ND, INCOMPLETELY OSSIFIED PELVIS: ISCHIUM, INCOMPLETELY OSSIFIED, bilateral
							FETUS 7 PELVIS: ISCHIUM, INCOMPLETELY OSSIFIED, right
							FETUS 14 STERNAL CENTRA: 1ST, INCOMPLETELY OSSIFIED; 2ND, INCOMPLETELY OSSIFIED PELVIS: ISCHIUM, INCOMPLETELY OSSIFIED, bilateral
6863	1(6.2)	0/16		0/ 8		1/ 8	FETUS 13 STERNAL CENTRA: 1ST, INCOMPLETELY OSSIFIED

N/N = NUMBER OF SPECIMENS WITH ALTERATIONS/NUMBER OF SPECIMENS EXAMINED

PROTOCOL 720-002: DEVELOPMENTAL TOXICITY STUDY OF HYAMINE® 1622 IN RATS

TABLE 20 (PAGE 15): FETAL ALTERATIONS - INDIVIDUAL DATA

DOSAGE GROUP III		30 MG/KG/DAY					
RAT NUMBER	SPECIMENS WITH ANY ALTERATIONS N(%)	GROSS EXTERNAL EXAMINATION		SOFT TISSUE EXAMINATION		SKELETAL EXAMINATION	
		N/N	DESCRIPTION	N/N	DESCRIPTION	N/N	DESCRIPTION
6864	1(6.2)	0/16		0/ 8		1/ 8	FETUS 9 PELVIS: PUBIS, INCOMPLETELY OSSIFIED, bilateral
6865	5(35.7)	0/14		0/ 7		5/ 7	FETUS 3 RIBS: WAVY, right 8th, 9th and 12th; INCOMPLETELY OSSIFIED (HYPOPLASTIC), right 8th, 9th and 12th FETUS 5 RIBS: WAVY, bilateral 4th - 12th; INCOMPLETELY OSSIFIED (HYPOPLASTIC), bilateral 8th - 10th FETUS 7 RIBS: WAVY, bilateral 4th - 12th; INCOMPLETELY OSSIFIED (HYPOPLASTIC), right 8th FETUS 11 RIBS: WAVY, right 6th, 7th and 11th

N/N = NUMBER OF SPECIMENS WITH ALTERATIONS/NUMBER OF SPECIMENS EXAMINED

PROTOCOL 720-002: DEVELOPMENTAL TOXICITY STUDY OF HYAMINE® 1622 IN RATS

TABLE 20 (PAGE 16): FETAL ALTERATIONS - INDIVIDUAL DATA

DOSAGE GROUP III		30 MG/KG/DAY					
RAT NUMBER	SPECIMENS WITH ANY ALTERATIONS N(%)	GROSS EXTERNAL EXAMINATION		SOFT TISSUE EXAMINATION		SKELETAL EXAMINATION	
		N/N	DESCRIPTION	N/N	DESCRIPTION	N/N	DESCRIPTION
6865 (cont.)							FETUS 13 THORACIC VERTEBRAE: CENTRUM, BIFID, 5th
6866	1(6.7)	0/15		1/ 7	FETUS 13 UMBILICAL ARTERY: DESCENDS TO THE LEFT OF URINARY BLADDER	0/ 8	
6867	1(7.1)	0/14		0/ 7		1/ 7	FETUS 9 THORACIC VERTEBRAE: CENTRUM, BIFID, 11th
6868	0(0.0)	0/12		0/ 6		0/ 6	
6869	1(6.7)	0/15		0/ 7		1/ 8	FETUS 7 STERNAL CENTRA: 1ST, INCOMPLETELY OSSIFIED
6870	1(10.0)	0/10		0/ 5		1/ 5	FETUS 10 RIBS: WAVY, right 8th - 11th
6871	0(0.0)	0/15		0/ 7		0/ 8	
6872	1(7.7)	0/13		0/ 6		1/ 7	FETUS 14 THORACIC VERTEBRAE: CENTRUM, BIFID, 10th

N/N = NUMBER OF SPECIMENS WITH ALTERATIONS/NUMBER OF SPECIMENS EXAMINED

PROTOCOL 720-002: DEVELOPMENTAL TOXICITY STUDY OF HYAMINE® 1622 IN RATS

TABLE 20 (PAGE 17): FETAL ALTERATIONS - INDIVIDUAL DATA

DOSAGE GROUP III		30 MG/KG/DAY					
RAT NUMBER	SPECIMENS WITH ANY ALTERATIONS N(%)	GROSS EXTERNAL EXAMINATION		SOFT TISSUE EXAMINATION		SKELETAL EXAMINATION	
		N/N	DESCRIPTION	N/N	DESCRIPTION	N/N	DESCRIPTION
6873	3(20.0)	0/15		0/ 7		3/ 8	FETUS 5 THORACIC VERTEBRAE: CENTRUM, BIFID, 11th FETUS 7 THORACIC VERTEBRAE: CENTRUM, BIFID, 12th FETUS 15 STERNAL CENTRA: 1ST, INCOMPLETELY OSSIFIED
6874	5(33.3)	0/15		0/ 7		5/ 8	FETUS 1 STERNAL CENTRA: 1ST, INCOMPLETELY OSSIFIED FETUS 7 STERNAL CENTRA: 1ST, NOT OSSIFIED FETUS 9 STERNAL CENTRA: 1ST, INCOMPLETELY OSSIFIED PELVIS: PUBIS, INCOMPLETELY OSSIFIED, bilateral FETUS 14 PELVIS: PUBIS, INCOMPLETELY OSSIFIED, left FETUS 16 STERNAL CENTRA: 1ST, NOT OSSIFIED
6875	0(0.0)	0/15		0/ 7		0/ 8	

N/N = NUMBER OF SPECIMENS WITH ALTERATIONS/NUMBER OF SPECIMENS EXAMINED

PROTOCOL 720-002: DEVELOPMENTAL TOXICITY STUDY OF HYAMINE® 1622 IN RATS

TABLE 20 (PAGE 18): FETAL ALTERATIONS - INDIVIDUAL DATA

DOSAGE GROUP IV		100 MG/KG/DAY					
RAT NUMBER	SPECIMENS WITH ANY ALTERATIONS N(%)	GROSS EXTERNAL EXAMINATION		SOFT TISSUE EXAMINATION		SKELETAL EXAMINATION	
		N/N	DESCRIPTION	N/N	DESCRIPTION	N/N	DESCRIPTION
6876	0(0.0)	0/15		0/ 7		0/ 8	
6877	0(0.0)	0/13		0/ 6		0/ 7	
6878	0(0.0)	0/14		0/ 7		0/ 7	
6879	0(0.0)	0/13		0/ 6		0/ 7	
6880	0(0.0)	0/14		0/ 7		0/ 7	
6881	NOT PREGNANT						
6882	0(0.0)	0/11		0/ 5		0/ 6	
6883	0(0.0)	0/14		0/ 7		0/ 7	
6884	0(0.0)	0/14		0/ 7		0/ 7	
6885	1(11.1)	1/ 9	FETUS 6 EYES: BULGE DEPRESSED, left	0/ 4		1/ 5	FETUS 6 SKULL: EYE SOCKET, SMALL, left CERVICAL VERTEBRAE: CERVICAL RIB AT 7TH CERVICAL VERTEBRA, left STERNAL CENTRA: 1ST, INCOMPLETELY OSSIFIED PELVIS: PUBIS, INCOMPLETELY OSSIFIED, left

N/N = NUMBER OF SPECIMENS WITH ALTERATIONS/NUMBER OF SPECIMENS EXAMINED

PROTOCOL 720-002: DEVELOPMENTAL TOXICITY STUDY OF HYAMINE® 1622 IN RATS

TABLE 20 (PAGE 19): FETAL ALTERATIONS - INDIVIDUAL DATA

DOSAGE GROUP IV		100 MG/KG/DAY					
RAT NUMBER	SPECIMENS WITH ANY ALTERATIONS N(%)	GROSS EXTERNAL EXAMINATION		SOFT TISSUE EXAMINATION		SKELETAL EXAMINATION	
		N/N	DESCRIPTION	N/N	DESCRIPTION	N/N	DESCRIPTION
6886	3(21.4)	0/14		0/ 7		3/ 7	FETUS 5 PELVIS: PUBIS, INCOMPLETELY OSSIFIED, bilateral FETUS 9 STERNAL CENTRA: 1ST, INCOMPLETELY OSSIFIED FETUS 15 THORACIC VERTEBRAE: CENTRUM, BIFID, 11th
6887	0(0.0)	0/12		0/ 6		0/ 6	
6888	1(5.9)	0/17		0/ 8		1/ 9	FETUS 5 STERNAL CENTRA: 1ST, NOT OSSIFIED
6889	0(0.0)	0/16		0/ 8		0/ 8	
6890	2(15.4)	1/13	FETUS 10 EYES: BULGE DEPRESSED, left a	1/ 6	FETUS 10 EYES: MICROPTHALMIA, left	1/ 7	FETUS 7 CERVICAL VERTEBRAE: CERVICAL RIB AT 7TH CERVICAL VERTEBRA, left

N/N = NUMBER OF SPECIMENS WITH ALTERATIONS/NUMBER OF SPECIMENS EXAMINED

a. First observed at soft tissue examination.

PROTOCOL 720-002; DEVELOPMENTAL TOXICITY STUDY OF HYAMINE® 1622 IN RATS

TABLE 20 (PAGE 20); FETAL ALTERATIONS - INDIVIDUAL DATA

DOSAGE GROUP IV		100 MG/KG/DAY					
RAT NUMBER	SPECIMENS WITH ANY ALTERATIONS N(%)	GROSS EXTERNAL EXAMINATION		SOFT TISSUE EXAMINATION		SKELETAL EXAMINATION	
		N/N	DESCRIPTION	N/N	DESCRIPTION	N/N	DESCRIPTION
6891	0(0.0)	0/14	FETUS 14 LATE RESORPTION, autolysis precluded further evaluation	0/ 7		0/ 7	
6892	0(0.0)	0/16		0/ 8		0/ 8	
6893	1(7.7)	0/13		0/ 6		1/ 7	FETUS 8 PELVIS: PUBIS, INCOMPLETELY OSSIFIED, bilateral
6894	1(7.1)	0/14		0/ 7		1/ 7	FETUS 7 LUMBAR VERTEBRAE: CENTRUM, UNILATERAL OSSIFICATION, left 1st
6895	1(6.7)	0/15	FETUS 1 LATE RESORPTION, autolysis precluded further evaluation	0/ 7		1/ 8	FETUS 3 THORACIC VERTEBRAE: HEMIVERTEBRA, right 4th, centrum and arch with attached rib; CENTRUM, BIFID, 2nd, 6th, 7th, 8th and 10th; CENTRA, FUSED, left 7th to right 8th
6896	0(0.0)	0/14		0/ 7		0/ 7	
6897	0(0.0)	0/15		0/ 7		0/ 8	
6898	0(0.0)	0/16		0/ 8		0/ 8	

N/N = NUMBER OF SPECIMENS WITH ALTERATIONS/NUMBER OF SPECIMENS EXAMINED

PROTOCOL 720-002: DEVELOPMENTAL TOXICITY STUDY OF HYAMINE® 1622 IN RATS

TABLE 20 (PAGE 21): FETAL ALTERATIONS - INDIVIDUAL DATA

DOSAGE GROUP IV		100 MG/KG/DAY					
RAT NUMBER	SPECIMENS WITH ANY ALTERATIONS N(%)	GROSS EXTERNAL EXAMINATION		SOFT TISSUE EXAMINATION		SKELETAL EXAMINATION	
		N/N	DESCRIPTION	N/N	DESCRIPTION	N/N	DESCRIPTION
6899	0(0.0)	0/14		0/ 7		0/ 7	
6900	1(6.7)	0/15		0/ 7		1/ 8	FETUS 9 PELVIS: PUBIS, INCOMPLETELY OSSIFIED, left

N/N = NUMBER OF SPECIMENS WITH ALTERATIONS/NUMBER OF SPECIMENS EXAMINED

PROTOCOL 720-002: DEVELOPMENTAL TOXICITY STUDY OF HYAMINE® 1622 IN RATS

TABLE 20 (PAGE 22): FETAL ALTERATIONS - INDIVIDUAL DATA

DOSAGE GROUP V		170 MG/KG/DAY					
RAT NUMBER	SPECIMENS WITH ANY ALTERATIONS N(%)	GROSS EXTERNAL EXAMINATION		SOFT TISSUE EXAMINATION		SKELETAL EXAMINATION	
		N/N	DESCRIPTION	N/N	DESCRIPTION	N/N	DESCRIPTION
6901	0(0.0)	0/12		0/ 6		0/ 6	
6902	0(0.0)	0/14		0/ 7		0/ 7	
6903	1(5.6)	0/18		0/ 9		1/ 9	FETUS 3 THORACIC VERTEBRAE: CENTRUM, BIFID, 7th
6904	0(0.0)	0/15		0/ 7		0/ 8	
6905	0(0.0)	0/17		0/ 8		0/ 9	
6906	0(0.0)	0/14		0/ 7		0/ 7	
6907	0(0.0)	0/ 5		0/ 2		0/ 3	
6908	0(0.0)	0/11		0/ 5		0/ 6	
6909	0(0.0)	0/12		0/ 6		0/ 6	
6910	FOUND DEAD ON DAY 11 OF GESTATION						
6911	0(0.0)	0/13		0/ 6		0/ 7	
6912	2(20.0)	0/10		0/ 5		2/ 5	FETUS 5 PELVIS: PUBIS, INCOMPLETELY OSSIFIED, bilateral

N/N = NUMBER OF SPECIMENS WITH ALTERATIONS/NUMBER OF SPECIMENS EXAMINED

PROTOCOL 720-002: DEVELOPMENTAL TOXICITY STUDY OF HYAMINE® 1622 IN RATS

TABLE 20 (PAGE 23): FETAL ALTERATIONS - INDIVIDUAL DATA

DOSAGE GROUP V		170 MG/KG/DAY					
RAT NUMBER	SPECIMENS WITH ANY ALTERATIONS N(%)	GROSS EXTERNAL EXAMINATION		SOFT TISSUE EXAMINATION		SKELETAL EXAMINATION	
		N/N	DESCRIPTION	N/N	DESCRIPTION	N/N	DESCRIPTION
6912 (cont.)							FETUS 7 PELVIS: ISCHIUM, INCOMPLETELY OSSIFIED, bilateral; PUBIS, INCOMPLETELY OSSIFIED, right
6913	0(0.0)	0/14		0/ 7		0/ 7	
6914	0(0.0)	0/14		0/ 7		0/ 7	
6915	4(26.7)	0/15		0/ 7		4/ 8	FETUS 1 STERNAL CENTRA: 1ST, NOT OSSIFIED FETUS 3 RIBS: WAVY, right 5th - 12th PELVIS: PUBIS, INCOMPLETELY OSSIFIED, bilateral FETUS 10 RIBS: WAVY, right 4th - 11th; INCOMPLETELY OSSIFIED (HYPOPLASTIC), right 8th and 11th PELVIS: ISCHIUM, INCOMPLETELY OSSIFIED, bilateral

N/N = NUMBER OF SPECIMENS WITH ALTERATIONS/NUMBER OF SPECIMENS EXAMINED

PROTOCOL 720-002: DEVELOPMENTAL TOXICITY STUDY OF HYAMINE® 1622 IN RATS

TABLE 20 (PAGE 24): FETAL ALTERATIONS - INDIVIDUAL DATA

DOSAGE GROUP V		170 MG/KG/DAY					
RAT NUMBER	SPECIMENS WITH ANY ALTERATIONS N(%)	GROSS EXTERNAL EXAMINATION		SOFT TISSUE EXAMINATION		SKELETAL EXAMINATION	
		N/N	DESCRIPTION	N/N	DESCRIPTION	N/N	DESCRIPTION
6915 (cont.)							FETUS 12 STERNAL CENTRA: 1ST, NOT OSSIFIED PELVIS: ISCHIUM, INCOMPLETELY OSSIFIED, left; PUBIS, INCOMPLETELY OSSIFIED, bilateral
6916	2(15.4)	0/13		0/ 6		2/ 7	FETUS 10 STERNAL CENTRA: 1ST, INCOMPLETELY OSSIFIED FETUS 12 RIBS: WAVY, right 5th, 9th - 12th, left 5th and 6th
6917	3(37.5)	0/ 8		0/ 4		3/ 4	FETUS 4 STERNAL CENTRA: 1ST, INCOMPLETELY OSSIFIED FETUS 7 STERNAL CENTRA: 1ST, NOT OSSIFIED FETUS 9 STERNAL CENTRA: 1ST, NOT OSSIFIED
6918	FOUND DEAD ON DAY 13 OF GESTATION						
6919	0(0.0)	0/ 6		0/ 3		0/ 3	

N/N = NUMBER OF SPECIMENS WITH ALTERATIONS/NUMBER OF SPECIMENS EXAMINED

PROTOCOL 720-002: DEVELOPMENTAL TOXICITY STUDY OF HYAMINE® 1622 IN RATS

TABLE 20 (PAGE 25): FETAL ALTERATIONS - INDIVIDUAL DATA

DOSAGE GROUP V		170 MG/KG/DAY					
RAT NUMBER	SPECIMENS WITH ANY ALTERATIONS N(%)	GROSS EXTERNAL EXAMINATION		SOFT TISSUE EXAMINATION		SKELETAL EXAMINATION	
		N/N	DESCRIPTION	N/N	DESCRIPTION	N/N	DESCRIPTION
6920	1(7.1)	1/14	FETUS 7 TAIL: ABSENT	0/ 7		1/ 7	FETUS 7 SACRAL VERTEBRAE: 2 PRESENT a CAUDAL VERTEBRAE: 0 PRESENT a
6921	FOUND DEAD ON DAY 16 OF GESTATION						
6922	2(14.3)	0/14		0/ 7		2/ 7	FETUS 7 RIBS: WAVY, right 11th and 12th FETUS 9 RIBS: WAVY, right 4th - 12th, left 6th - 12th; INCOMPLETELY OSSIFIED (HYPOPLASTIC), right 9th - 12th, left 10th - 12th
6923	FOUND DEAD ON DAY 12 OF GESTATION						
6924	2(15.4)	0/13		0/ 6		2/ 7	FETUS 11 STERNAL CENTRA: 1ST, INCOMPLETELY OSSIFIED FETUS 15 THORACIC VERTEBRAE: CENTRUM, BIFID, 12th

N/N = NUMBER OF SPECIMENS WITH ALTERATIONS/NUMBER OF SPECIMENS EXAMINED

a. Excluded from group averages and statistical analyses.

PROTOCOL 720-002: DEVELOPMENTAL TOXICITY STUDY OF HYAMINE® 1622 IN RATS

TABLE 20 (PAGE 26): FETAL ALTERATIONS - INDIVIDUAL DATA

DOSAGE GROUP V		170 MG/KG/DAY					
RAT NUMBER	SPECIMENS WITH ANY ALTERATIONS N(%)	GROSS EXTERNAL EXAMINATION		SOFT TISSUE EXAMINATION		SKELETAL EXAMINATION	
		N/N	DESCRIPTION	N/N	DESCRIPTION	N/N	DESCRIPTION
6925	2(11.1)	0/18		0/ 9		2/ 9	FETUS 1 PELVIS: PUBIS, INCOMPLETELY OSSIFIED, left FETUS 11 STERNAL CENTRA: 1ST, NOT OSSIFIED PELVIS: PUBIS, INCOMPLETELY OSSIFIED, bilateral

N/N = NUMBER OF SPECIMENS WITH ALTERATIONS/NUMBER OF SPECIMENS EXAMINED

APPENDIX 1
PROTOCOL AND AMENDMENT



Argus Research Laboratories, Inc.
905 Sheehy Drive, Building A
Horsham, Pennsylvania 19044
T: (215) 443-8710 F: (215) 443-8587

PROTOCOL 720-002

STUDY TITLE: Developmental Toxicity Study of Hyamine® 1622 in Rats

PURPOSE: The purpose of this study is to evaluate the potential for Hyamine® 1622 to produce developmental toxicity (embryo-fetal toxicity and teratogenesis) when administered orally via gavage to Crl:CD®BR VAF/Plus® presumed pregnant rats.

TESTING FACILITY: Argus Research Laboratories, Inc.
905 Sheehy Drive, Building A
Horsham, Pennsylvania 19044-1297
Telephone: (215) 443-8710
Telefax: (215) 443-8587

STUDY DIRECTOR: John A. Foss, Ph.D.
Group Leader

SPONSOR: Lonza Inc.
17-17 Route 208
Fair Lawn, New Jersey 07410

**SPONSOR'S
REPRESENTATIVE:** Michael W. Gill, Ph.D.
Toxicology/Regulatory Services
2345 Hunters Way #3
Charlottesville, Virginia 22901
Telephone: (804) 977-5957
Telefax: (804) 977-0899

REGULATORY CITATIONS:

U.S. Environmental Protection Agency (1984). *Pesticide Assessment Guidelines*. Subdivision F - Hazard Evaluation: Human and Domestic Animals, November, 1984 (Revised Edition).

U.S. Environmental Protection Agency. Federal Insecticide, Fungicide and Rodenticide Act (FIFRA); Good Laboratory Practice Standards; Final Rule. 40 CFR Part 160.

REGULATORY COMPLIANCE

This study will be conducted in compliance with the Good Laboratory Practice (GLP) regulations cited above.

No changes will be made to this protocol without the specific written request or consent of the Sponsor's Representative. The Sponsor's Representative may request a change verbally. However, it then becomes the responsibility of the Sponsor's Representative to follow such a verbal request with written verification. The Testing Facility reserves the right to deviate from the protocol without prior approval from the Sponsor's Representative if the integrity of the study is in jeopardy. In that case, the Study Director will notify the Sponsor Representative as soon as possible. All changes or revisions of this protocol shall be documented, signed by the Study Director and the Sponsor's Representative, dated and maintained with the protocol.

The Quality Assurance Unit will audit the protocol, the raw data and the draft and final report, and will inspect critical phases of the study in accordance with the Standard Operating Procedures of Argus Research Laboratories, Inc.

The final report will include a statement signed by the Study Director that the report accurately reflects the raw data obtained during the performance of the study and that all applicable Good Laboratory Practice (GLP) regulations were followed in the conduct of the study. Should significant deviations from GLP regulations occur, each will be described in detail, together with how the deviation might affect the quality or integrity of the study.

SCHEDULE:

See ATTACHMENT 1 to the protocol.

TEST SUBSTANCE AND VEHICLE:

Identification:

Test Substance:

Chemical Name: Benzethonium Chloride
 Synonym: Hyamine® 1622
 Source: Lonza Inc., 79 Route 22 East, Annandale, New Jersey 08801
 CAS Registry Number: 121-54-0
 Sponsor ID: Hyamine® 1622 (Lonza TRCS Number: 40109)
 Argus Research Laboratories, Inc. ID: Hyamine® 1622
 Percent Active Ingredient: 99.3
 Description: Homogenous, fine, white powder free from visible foreign matter

Vehicle:

Reverse osmosis processed deionized water (R.O. deionized water).

Neither the Sponsor nor the Study Director is aware of any potential contaminants likely to be present in the vehicle that would interfere with the results of this study. Therefore, no analyses other than those mentioned in this protocol will be conducted.

Testing Facility Reserve Samples:

The Testing Facility will reserve a 10 g sample of each lot of bulk test substance and vehicle used during the course of the study. Reserve samples will be stored under the conditions cited below (see Storage).

Safety Precautions:

Gloves, mask, appropriate eye protection and uniform/lab coat to be worn during formulation preparation and dosage. The Material Safety Data Sheet is attached to the protocol (see ATTACHMENT 2).

Storage:

Bulk Test Substance: Ambient temperature in an environmentally controlled area.
 Prepared Formulations: Ambient temperature in an environmentally controlled area.

Stability:

The test substance is considered to be stable at room temperature for the duration of the study.

FORMULATION:**Preparation of Dosing Solutions:**

Each dosing solution will be prepared by combining the appropriate amounts of the test substance (grams) and R.O. deionized water to obtain the required volume of solution. Detailed preparation procedures are attached to this protocol (ATTACHMENT 3).

Dosing solutions will be prepared once for the study, unless the stability analysis indicates that the solutions are not stable for at least 21 days after preparation. Before dosage each day, aliquots of the dosing solutions will be transferred to appropriate containers for daily dosage. Each container will be weighed after adding the daily aliquot, at the end of dosage, and after the remaining portion of each aliquot is discarded in order to determine the amounts of solutions that are used for dosage and that are discarded each day.

Adjustment for Purity:

The amount of the test substance used in the preparation of the dosing solutions will not be adjusted for percent active ingredient since the test substance is >99% pure.

ANALYSES OF DOSING SOLUTIONS:

Dosing solutions will be analyzed for the concentration of test substance at Lancaster Laboratories, Inc., Lancaster, Pennsylvania.

Homogeneity, Stability and Concentration Analyses:

Homogeneity and stability will be evaluated for concentrations of 0.3, 15 and 20 mg/mL. One sample (6 mL) each will be taken on the day of preparation from the top, middle and bottom of the mixing vessel for each concentration to evaluate homogeneity. The solutions will then be stored at room temperature and additional samples (6 mL; one each from the top, middle and bottom of the vessel for each concentration) will be taken at 14 days and again at 21 days after preparation to evaluate the stability and longer term homogeneity of the dosing solutions. Each sample will be divided into three approximately equal parts (2 mL each); two parts will be refrigerated (2°C to 8°C) and shipped for analysis, and one part will be retained refrigerated at the Test Facility as a backup.

Dosing solutions prepared for administration to the rats in this study will be analyzed for the concentration of test substance before being administered to the rats. One sample (6 mL) will be taken from the middle of each concentration on the day of preparation. Each sample will be divided into three approximately equal parts (2 mL each); two parts will be refrigerated and shipped for analysis, and one part will be retained refrigerated at the Test Facility as a backup.

The standards for acceptable accuracy of mixing will be: 1) the mean of the analyzed samples must be within $\pm 10\%$ of nominal; 2) the difference between duplicate analyses must not exceed $\pm 10\%$; and 3) individual analyses must be $\pm 15\%$ of nominal. If one or more of these standards are not met, the solutions will not be administered to the rats. If additional analyses or solution preparations are necessary, these will be performed at no additional cost to the Sponsor. The Study Director and Sponsor's Representative will be notified immediately when problem of this nature occur.

Shipping Instructions:

Portions of the samples to be analyzed will be shipped (on wet ice) to:

Gloria Gates, B.S.
Lancaster Laboratories, Inc.
2425 New Holland Pike
Lancaster, Pennsylvania 17601
Telephone: (717) 656-2301

The recipient will be notified in advance of sample shipment.

DISPOSITION:

Prepared formulations and portions of samples of prepared formulations retained at the Test Facility will be discarded at the Testing Facility at the end of the study following consultation with the Sponsor's Representative. All remaining bulk test substance will be returned to the Sponsor after the study(ies) with this test substance have been completed and the Test Facility has received the written consent of the Sponsor's Representative.

TEST SYSTEM:

Species/Strain and Reason for Selection:

The CrI:CD®BR VAF/Plus® (Sprague-Dawley) rat was selected as the Test System because: 1) it is one mammalian species accepted and widely used throughout industry for nonclinical studies of developmental toxicity (embryo-fetal toxicity/teratogenicity); 2) this strain has been demonstrated to be sensitive to

developmental toxins; and 3) historical data and experience exist at the Testing Facility⁽¹⁻³⁾.

Number:

Initial population acclimated: 165 virgin female rats.
Population selected for study: 125 mated female rats (25 per dosage group).

Body Weight and Age:

Female rats will be ordered to have body weights of 225 g to 250 g each at receipt, at which time they will be expected to be at least 60 days of age. Actual body weights recorded at receipt will be documented in the raw data, and the weight range will be included in the final report.

Sex:

Female rats will be given the test substance. Male rats of the same source and strain will be used only as breeders and are not considered part of the Test System.

Source:

Charles River Laboratories, Inc., Portage, Michigan.

The rats will be shipped in filter-topped cartons by truck directly from Charles River Laboratories, Inc., to the Testing Facility.

Identification:

Rats will be permanently identified using Monel® self-piercing ear tags (Gey Band and Tag Co., Inc., No. MSPT 20101). Male rats will be given unique permanent identification numbers upon assignment to the Testing Facility's breeder male rat population. Female rats will be assigned temporary numbers at receipt, and given unique permanent identification numbers when assigned to study on the basis of day 0 of presumed gestation body weights.

Prestudy Health Screen:

Within three days after arrival, five female rats will be randomly selected (with the restriction that no more than one rat will be selected from any one shipping crate) from the initial population of animals received at the Testing Facility and examined by a Staff Veterinarian for general health condition. Fecal samples will be collected from each of the five rats and examined for parasites. The selected rats will be sacrificed (carbon dioxide asphyxiation) and a blood sample will be collected from the vena cava. The blood will be centrifuged and the resulting serum will be diluted 1:4 with phosphate buffered saline. The serum dilution will be shipped (on dry ice) to Charles

River Laboratories, Inc. [Diagnostic Lab, 251 Ballardvale Street, Wilmington, Massachusetts 01887, Telephone: (508) 658-6000] for routine serology testing. A gross necropsy of the thoracic, abdominal and pelvic viscera will be performed. Gross lesions will be retained in neutral buffered 10% formalin for possible further evaluation. Results of this testing will be maintained in the raw data and reported to the Sponsor's Representative, who will notify the Testing Facility of the acceptability of the rats before the start of cohabitation.

In addition, male rats comprising the Testing Facility breeder male rat colony will be examined by a veterinarian before mating. Also, three male rats from this breeder male rat colony will be selected for fecal parasite evaluations. The results of the fecal parasite screen and the veterinarian examination will be reported to the Sponsor's Representative before the cohabitation period begins.

ANIMAL HUSBANDRY:

All cage sizes and housing conditions are in compliance with the *Guide for the Care and Use of Laboratory Animals*⁽⁴⁾. Animal care procedures will be performed according to the Testing Facility Standard Operating Procedures (SOPs).

Housing:

With the exception of the cohabitation period, rats will be individually housed in stainless-steel wire-bottomed cages. During cohabitation, each pair of rats will be placed in one cage. No nesting materials will be supplied because the female rats will be sacrificed before the expected day of natural delivery.

Room Air, Temperature and Humidity:

The animal room is independently supplied with at least ten changes per hour of 100% fresh air that has been passed through 99.97% HEPA filters (Airo Clean® room). Room temperature will be maintained at 70°F to 78°F and monitored constantly. Room humidity will also be monitored constantly and maintained at 40% to 70%.

Light:

An automatically-controlled 12-hour light:12-hour dark fluorescent light cycle will be maintained. Each dark period will begin at 1900 hours EST.

Diet:

Rats will be given Certified Rodent Diet® #5002 (Purina Mills, Inc.) available *ad libitum* from individual feeders. Individual feeders will be changed at least weekly and will be checked daily to ensure *ad libitum* availability of feed. Documentation of these procedures will be maintained in the raw data.

Water:

Water will be available *ad libitum* from an automatic watering access system (individual water bottles will also be attached to cages if deemed necessary). All water will be from a local source and passed through a reverse osmosis membrane before use. Chlorine will be added to the processed water as a bacteriostat; processed water is expected to contain no more than 1.2 ppm chlorine at the time of analysis. Water is analyzed monthly for possible bacterial contamination and twice annually for possible chemical contamination.

Contaminants:

Neither the Sponsor nor the Study Director is aware of any potential contaminants likely to be present in the certified diet or in the drinking water that would interfere with the results of this study. Therefore, no analyses other than those routinely performed by the feed supplier or those mentioned in this protocol will be conducted.

ACCLIMATION:

The rats will be acclimated for two weeks before cohabitation. During this period, each rat will be checked for viability and overt signs of toxicity at least twice daily; once in the morning and once in the afternoon, at least four (4) hours apart. Overt signs of toxicity will be recorded for individual rats when observed. Detailed examination for clinical signs of disease or abnormality, which involve handling of the rat outside of its cage, will be conducted, and observations of both the presence or absence of clinical signs recorded, on four occasions: on Tuesday and Friday during the week of arrival at the Testing Facility; on the following Friday; and on the following Monday when the rats are placed into cohabitation. Body weights will be recorded at a consistent time of day [between 0800 and 1100 (EST)] on the same days that clinical observations are conducted.

Prior to cohabitation each rat will also be examined by the Staff Veterinarian for general health condition. Only rats that appear healthy and have normal weight gain during the acclimation period will be selected for cohabitation.

RANDOMIZATION AND COHABITATION:

Upon arrival, male and female rats will be assigned to individual housing on the basis of computer-generated random units.

After acclimation, virgin female rats will be placed in cohabitation with breeder male rats, one male rat per female rat. The cohabitation period will consist of a maximum of five days. Rats with spermatozoa observed in a smear of the vaginal contents and/or a copulatory plug observed *in situ* will be considered to be at day 0 of presumed gestation and assigned to individual housing.

Healthy mated female rats will be assigned to study groups using a second computer-generated (weight-ordered) randomization procedure.

ADMINISTRATION:

Route and Reason for Choice:

The oral (gavage) route was selected for use because: 1) the oral route is one possible route of human exposure; and 2) the daily dosage can be accurately administered using the gavage method.

Method and Frequency:

The female rats will be given the test substance once daily on days 6 through 15 of presumed gestation, the period of organogenesis. The test substance will be administered as a single daily dose using a 2- or 3-inch long 16- or 18-gauge stainless-steel gavage needle attached to a 5 cc disposable syringe. The dosage volume in all groups will be 10 mL/kg/day with the actual volume based on the most recent body weight of each rat. Administration will be at approximately the same time each day.

Rationale for Dosage Selection:

Dosages were selected on the basis of the results of a developmental toxicity dose range-finding study with this test substance in rats (Argus Research Laboratories, Inc., Protocol 720-002P).

Dosage Levels, Concentrations and Volumes:

Group	Number of Rats	Dosage (mg/kg/day)*	Concentration (mg/mL)*	Volume (mL/kg)	Argus Batch Number
I	25	0	0	10	B-720-002-A(Day.Month.Year)
II	25	10	1.0	10	B-720-002-B(Day.Month.Year)
III	25	30	3.0	10	B-720-002-C(Day.Month.Year)
IV	25	100	10.0	10	B-720-002-D(Day.Month.Year)
V	25	170	17.0	10	B-720-002-E(Day.Month.Year)

- a. Dosage calculations will not be adjusted for the percent active ingredient since the test substance is >99% pure.

TESTS, ANALYSES AND MEASUREMENTS:**Viability/Overt Signs of Toxicity:**

All Periods: Each animal will be checked for viability and overt signs of toxicity at least twice daily; once in the morning and once in the afternoon, at least four (4) hours apart. Overt signs of toxicity will be recorded for individual rats when observed.

Clinical Observations and/or General Appearance:

Detailed examination for clinical signs of disease or abnormality, which involve handling of the rat outside of its cage, will be conducted according to the schedule listed below. A record of clinical observations or the absence of clinical signs will be made for each rat.

Acclimation Period: At least four times (see ACCLIMATION).

Predosage Period: Daily beginning on day 0 of presumed gestation.

Dosage Period: Daily before dosage. Postdosage observations will also be recorded approximately one hour after administration.

Postdosage Period: Once daily.

Clinical observations may be recorded more frequently than cited above, if deemed appropriate by the Study Director and/or Sponsor's Representative.

Body Weights (recorded and tabulated):

Body weights will be measured for individual rats at a consistent time of day [between 0800 and 1100 (EST)] according to the schedule listed below.

Acclimation Period: On Tuesday and Friday during the week of arrival at the Testing Facility; on the following Friday; and on the following Monday when the rats are placed into cohabitation (see ACCLIMATION).

Predosage Period: Day 0 of presumed gestation.

Dosage Period: Days 6, 9, 12 and 15 of presumed gestation.

Postdosage Period: Days 16, 18 and 20 of presumed gestation.

Feed Consumption Values (recorded and tabulated):

Feed consumption values will be measured for individual animals according to the schedule listed below.

Predosage Period: Day 0 of presumed gestation.

Dosage Period: Days 6, 9, 12 and 15 of presumed gestation.

Postdosage Period: Days 16, 18 and 20 of presumed gestation.

Feed consumption values may be recorded more frequently if it is necessary to replenish the feed. These intervals will not be tabulated.

Mating Performance:

Mating will be evaluated daily during the cohabitation period and confirmed by observation of spermatozoa in a smear of the vaginal contents and/or a copulatory plug observed *in situ*.

Caesarean-Sectioning Observations:

Rats will be Caesarean-sectioned on day 20 of presumed gestation. The uterus will be excised and the weight recorded. The fetuses will be removed from the uterus and placed in individual containers.

The rats will be examined for number and distribution of:

Corpora Lutea.

Implantation Sites.

Live and Dead Fetuses.

(A live fetus is defined as one that responds to stimuli; a dead fetus is defined as a term fetus that does not respond to stimuli and that is not markedly autolyzed; dead fetuses demonstrating marked to extreme autolysis are considered to be late resorptions.)

Early and Late Resorptions.

(A conceptus is defined as a late resorption if it is grossly evident that organogenesis has occurred; if this is not the case, the conceptus is defined as an early resorption.)

Fetal Observations:**Gross External Alterations and Sex:**

Fetuses will be examined for sex and for gross external alterations. Late resorptions and dead fetuses will be examined for gross external alterations to the extent possible, but such observations will not be included in either data summarization or statistical analyses.

Body Weights and Identification:

The body weight of each fetus (live and dead) will be recorded. Only body weights of live fetuses will be used in determining litter fetal body weight averages. Fetuses will be tagged with identification noting litter, uterine distribution, study number and fixative. Dead fetuses will be retained in neutral buffered 10% formalin for possible future evaluation.

Soft Tissue Examination:

Approximately one-half of the fetuses in each litter will be examined for soft tissue alterations using a variation of the microdissection technique of Staples⁽⁵⁾. The heads of these fetuses will be fixed in Bouin's solution and subsequently examined by free-hand sectioning; sections will be stored in alcohol. The remaining portion of each fetus (torso, limbs and tail) will be eviscerated and fixed in alcohol; skeletal preparations will be stained with alizarin red S⁽⁶⁾ and retained in glycerine for possible further examination.

Skeletal Examination:

Approximately one-half of the fetuses in each litter will be examined for skeletal alterations after staining with alizarin red S⁽⁶⁾. The fetuses will be initially fixed in alcohol; skeletal preparations will be retained in glycerine.

Representative photographs of fetal gross, soft tissue and skeletal alterations will be taken.

METHOD OF SACRIFICE:

Rats will be sacrificed by carbon dioxide asphyxiation. Fetuses will be sacrificed according to the Standard Operating Procedures of the Testing Facility.

NECROPSY:

Gross lesions will be retained in neutral buffered 10% formalin for possible future evaluation. Unless specifically cited below, all other tissues will be discarded.

Scheduled Sacrifice:

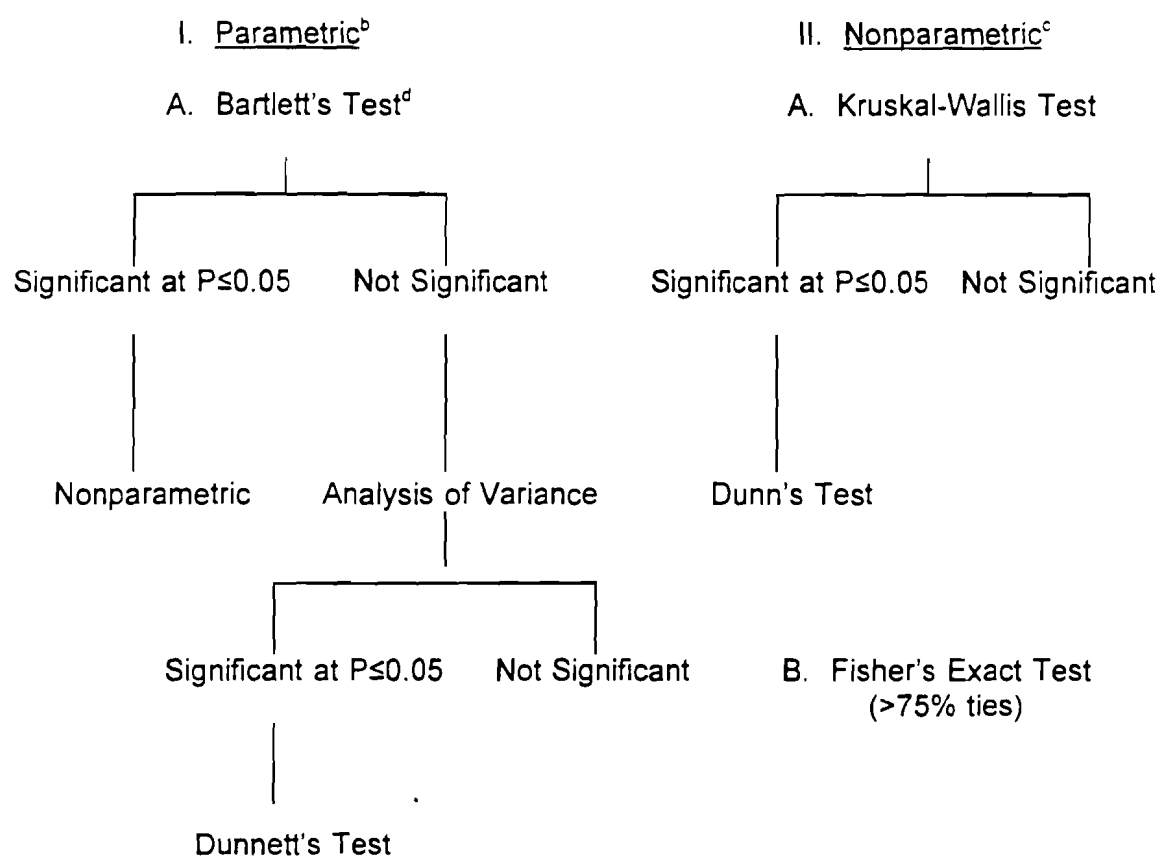
A gross necropsy of the thoracic, abdominal and pelvic viscera will be performed. Uteri of apparently nonpregnant rats will be stained with 10% ammonium sulfide and examined to determine pregnancy status⁽⁷⁾.

Rats Found Dead or Moribund:

Rats that die or are sacrificed because of moribund condition, abortion or delivery will be examined for the cause of death or moribund condition on the day the observation is made. The rats will be examined for gross lesions. Pregnancy status and uterine contents of female rats will be recorded. Uteri of apparently nonpregnant rats will be stained with 10% ammonium sulfide and examined to determine pregnancy status⁽⁷⁾.

PROPOSED STATISTICAL METHODS⁽⁸⁻¹⁴⁾:

Averages and percentages will be calculated. Litter values will be used where appropriate. Additional procedures and/or analyses may be performed, if appropriate.

Type of Test^a**III. Test for Proportion Data**

Variance Test for Homogeneity
of the Binomial Distribution

-
- a. All tests evaluated at P≤0.05 to P≤0.01.
 - b. Used only to analyze data with homogeneity of variance.
 - c. Proportion data are not included in this category.
 - d. Test for homogeneity of variance.

DATA ACQUISITION, VERIFICATION AND STORAGE:

Data will be hand- and/or computer-recorded. Generally, all raw data generated for a study are reviewed daily by a laboratory supervisor and weekly by the Study Director. Records will be reviewed by the Study Director and/or appropriate management personnel within 21 days after generation. All original records will be stored in the archives of the Testing Facility. All original data will be bound and indexed. A copy of all raw data will be supplied to the Sponsor upon request. Preserved tissues will be stored at the Testing Facility at no charge for one year after mailing of the draft final report, after which time the Sponsor will be contacted to determine the disposition of these materials.

RECORDS TO BE MAINTAINED:

Protocol and Amendments.
Test Substance, Vehicle and/or Reagent Receipt, Preparation and Use.
Animal Acquisition.
Randomization Schedules.
Prestudy Health Screen.
Mating History.
Treatment (if prescribed by Staff Veterinarian).
General Comments.
Results of Viability Checks and Examinations for Overt Signs of Toxicity.
Test Substance Administration.
Clinical Observations and/or General Appearance.
Body Weights.
Feed Consumption Values.
Caesarean-Sectioning and Fetal Observations.
Gross Necropsy Observations.
Organ Weights (if required).
Photographs (if required).
Study Maintenance (room and environmental records).
Feed and Water Analyses.
Packing and/or Shipment Lists.

KEY PERSONNEL:

Executive Director of Research: Mildred S. Christian, Ph.D., ATS
Director of Research: Alan M. Hoberman, Ph.D., DABT
Group Leader and Study Director: John A. Foss, Ph.D.
Director of Laboratory Operations: John F. Barnett, B.S.
Chairperson, Animal Care Committee: Denise E. Holliday, D.V.M.
Manager of Animal Operations and Member, Animal Care Committee:
Dena C. Lebo, V.M.D.
Manager of Regulatory Compliance: Kathleen A. Moran, B.A.

FINAL REPORT:

A comprehensive draft final report will be prepared on completion of the study and will be finalized following consultation with the Sponsor. The report will include the following:

- Summary and Conclusion.
- Experimental Design and Method.
- Evaluation of Test Results.
- Appendices: Figures, Summary and Individual Tables Summarizing the Above Data, Protocol and Associated Amendments and Deviations, Reports of Analyses of Test Substance Formulations, Study Director's GLP Compliance Statement, Reports of Supporting Data (if appropriate) and Quality Assurance Unit Statement.

ANIMAL CARE COMMITTEE STATEMENT:

The procedures described in this protocol have been reviewed by the Testing Facility's Animal Care Committee. All procedures described in this protocol that involve study animals will be conducted in a manner to avoid or minimize discomfort, distress or pain to the animals.

Information concerning the necessity for conducting this study and the fact that this is not an unnecessarily duplicative study may be obtained from the Sponsor. No alternative (*in vitro*) procedures were available for meeting the stated purposes of the study.

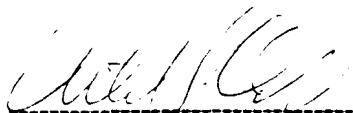
REFERENCES:

1. Christian, M.S. and Voytek, P.E. (1982). *In Vivo Reproductive and Mutagenicity Tests*. Environmental Protection Agency, Washington, D.C. National Technical Information Service, U.S. Department of Commerce, Springfield, VA 22161.
2. Christian, M.S. (1984). Reproductive toxicity and teratology evaluations of naltrexone (Proceedings of Naltrexone Symposium, New York Academy of Sciences, November 7, 1983), *J. Clin. Psychiat.* 45(9):7-10.
3. Lang, P.L. (1988). *Embryo and Fetal Developmental Toxicity (Teratology) Control Data in the Charles River CrI:CD®BR Rat*. Charles River Laboratories, Inc., Wilmington, MA 01887-0630. (Data base provided by Argus Research Laboratories, Inc.)

4. U.S. Department of Health and Human Services (1985). *Guide for the Care and Use of Laboratory Animals*. Prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, Commission on Life Sciences, National Research Council. Public Health Service, National Institutes of Health, NIH Publication No. 86-23.
5. Staples, R.E. (1974). Detection of visceral alterations in mammalian fetuses. *Teratology* 9(3):A37-38.
6. Modification of method of Staples, R.E. and Schnell, V.L. (1963). Refinement in rapid clearing technique in the KOH-alizarin red S method for fetal bone. *Stain Technol.* 39:61-63.
7. Salewski, E. (1964). Färbemethode zum makroskopischen Nachweis von Implantationsstellen am Uterus der Ratte. *Arch. Pathol. Exp. Pharmacol.* 247:367.
8. Snedecor, G.W. and Cochran, W.G. (1967). Variance test for homogeneity of the binomial distribution. *Statistical Methods*, 6th Edition, Iowa State University Press, Ames, pp. 240-241.
9. Sokal, R.R. and Rohlf, F.J. (1969). Bartlett's test of homogeneity of variances. *Biometry*, W.H. Freeman and Co., San Francisco, pp. 370-371.
10. Snedecor, G.W. and Cochran, W.G. (1967). Analysis of Variance. *Statistical Methods*, 6th Edition, Iowa State University Press, Ames, pp. 258-275.
11. Dunnett, C.W. (1955). A multiple comparison procedure for comparing several treatments with a control. *J. Amer. Stat. Assoc.* 50:1096-1129.
12. Sokal, R.R. and Rohlf, F.J. (1969). Kruskal-Wallis Test. *Biometry*, W.H. Freeman and Co., San Francisco, pp. 388-389.
13. Dunn, O.J. (1964). Multiple comparisons using rank sums. *Technometrics* 6(3):241-252.
14. Siegel, S. (1956). *Nonparametric Statistics for the Behavioral Sciences*, McGraw-Hill, New York, pp. 96-104.

PROTOCOL APPROVAL:

FOR THE TESTING FACILITY



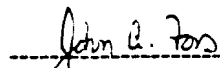
Mildred S. Christian, Ph.D., ATS
Executive Director of Research

19 May 95
Date



Alan M. Hoberman, Ph.D., DABT
Director of Research

18 May 95
Date



John A. Foss, Ph.D.
Group Leader and Study Director

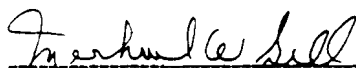
18 May 95
Date



Dena C. Lebo, V.M.D.
Member, Animal Care Committee

18 May 95
Date

FOR THE SPONSOR



Michael W. Gill, Ph.D.
Toxicology/Regulatory Services
Sponsor's Representative

5/24/95
Date

ATTACHMENT 1
SCHEDULE

SCHEDULE^a

08 MAY 95	Animals Arrive - Acclimation Begins.
22 MAY 95 PM - 27 MAY 95 AM	Cohabitation Period.
23 MAY 95	First Possible Day 0 of Presumed Gestation.
27 MAY 95	Last Possible Day 0 of Presumed Gestation.
29 MAY 95 - 11 JUN 95	Dosage Period (Days 6 through 15 of presumed gestation).
12 JUN 95 - 16 JUN 95	Caesarean-Sectioning Period (Day 20 of presumed gestation).
13 SEP 95	Draft Final Report.

a. The study initiation date is the date the Study Director signs the protocol.

ATTACHMENT 2
MATERIAL SAFETY DATA SHEET

MATERIAL SAFETY DATA

LONZA

17-17 Rt. 208
Fair Lawn, New Jersey 07410
800-777-1875 (9-5 P.M.)
309-697-5400 (After 5 P.M.)

EMERGENCY TELEPHONE
800-424-9300 (CHEMTREC)

REACTIVITY
F .BILITY

5970 Hyamine 1622 Crystals

PAGE 1 OF 6

MATERIAL	DATE ISSUED	DOT HAZARD CLASSIFICATION
Hyamine 1622 Crystals	11/12/92 - Rev.	Non-Hazardous
CAS NO. 121-54-0	SUPERCEDES	DOT SHIPPING NAME
	01/19/90	Not regulated
		DOT LABEL None

FORMULA $C_{27}H_{42}ClNO_2 \cdot H_2O$

CHEMICAL NAME Disobutylphenoxyethoxyethyl dimethylbenzylammonium chloride monohydrate

***** I - INGREDIENTS *****

	APPROXIMATE WEIGHT %	TWA/TLV
Diisobutylphenoxyethoxyethyl dimethylbenzyl- ammonium chloride monohydrate (CAS No. 121-54-0)	100	None established

***** II - PHYSICAL AND CHEMICAL PROPERTIES *****

APPEARANCE White free-flowing powder	pH Not applicable
VISCOSITY Not applicable	ODOR Odorless
BOILING POINT Not known	MELTING OR FREEZING POINT 329°F
VAPOR DENSITY (Air=1) Not applicable	VAPOR PRESSURE (mm Hg) Not applicable
PERCENT VOLATILE (by weight) Nil	SOLUBILITY IN WATER Soluble
EVAPORATION RATE (Butyl Acetate=1) Not applicable	BULK DENSITY 27.5 lb/ft ³

***** III - FIRE AND EXPLOSION INFORMATION *****

FLASH POINT Not known	AUTO IGNITION TEMPERATURE 716°F (cloud)		
LOWER EXPLOSION LIMIT (%) Not applicable	UPPER EXPLOSION LIMIT (%) Not applicable		
EXTINGUISHING MEDIA	FOAM	ALCOHOL FOAM	CO ₂ X
	DRY CHEMICAL X	WATER X	OTHER

SPECIAL FIRE FIGHTING PROCEDURES:

Must wear NIOSH/MSHA approved self-contained breathing apparatus and protective clothing. Cool fire-exposed containers with water spray.

UNUSUAL FIRE AND EXPLOSION HAZARDS:

Products of combustion are toxic.

As with most powdered organic compounds, dust explosions may be possible.

***** IV - HEALTH EFFECTS INFORMATION *****

ROUTES OF ENTRY - SKIN CONTACT X	EYE CONTACT X
INHALATION X	INGESTION

The information provided herein is compiled from internal reports and data from professional publications. IT IS FURNISHED WITHOUT WARRANTY OF ANY KIND, EXPRESS OR IMPLIED. It is intended to assist in evaluating the suitability and proper use of the material in manufacturing and in the development and implementation of safety precautions and procedures.

MATERIAL SAFETY DATA SHEET**LONZA**

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HEALTH	3
FLAMMABILITY	1
REACTIVITY	0

5970 Hyamine 1622 Crystals

PAGE 2 OF 6

***** IV - HEALTH EFFECTS INFORMATION (continued) *****

EFFECTS OF OVEREXPOSURE

Based upon the results of animal toxicity studies, it is anticipated that direct contact may produce severe skin and eye irritation.

OVEREXPOSURE MAY AGGRAVATE EXISTING CONDITIONS:

No effects indicated

EMERGENCY AND FIRST AID PROCEDURES:

Eyes: Flush eyes with large amounts of running water for at least 15 minutes. Hold eyelids apart to ensure rinsing of the entire surface of the eye and lids with water. Get immediate medical attention. If physician not available, flush for additional 15 minutes and then transport victim to medical care.

Skin: Wash with large amounts of running water, and soap if available, for 15 minutes. Remove contaminated clothing and shoes. Get immediate medical attention. Wash clothing and decontaminate shoes before reuse.

Ingestion: If swallowed, immediately give 3-4 glasses of milk (if unavailable, give water). DO NOT induce vomiting. If vomiting occurs, give fluids again. Get immediate medical attention. Have physician determine if patient's condition allows for induction of vomiting or evacuation of the stomach. Do not give anything by mouth to a convulsing or unconscious person.

Inhalation: Remove from area to fresh air. If not breathing, clear airway and start artificial respiration. If victim is having trouble breathing, give supplemental oxygen, if available. Get immediate medical attention.

CHEMICALS LISTED AS CARCINOGEN BY:

NATIONAL TOXICOLOGY PROGRAM	- No
I.A.R.C. MONOGRAPHS	- No
OSHA	- No

***** V - REACTIVITY INFORMATION *****

STABILITY:	STABLE	X	CONDITIONS TO AVOID
	UNSTABLE		None known

HAZARDOUS DECOMPOSITION PRODUCTS

Thermal decomposition may produce toxic vapors/fumes of amines and other organic materials, and oxides of carbon and nitrogen.

HAZARDOUS POLYMERIZATION

MAY OCCUR	WILL NOT X	CONDITIONS TO AVOID
	OCCUR	None known

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HEALTH	3
FLAMMABILITY	1
REACTIVITY	0

5970 Hyamine 1622 Crystals

PAGE 3 OF 6

***** V - REACTIVITY INFORMATION (continued) *****

INCOMPATIBILITY (MATERIALS TO AVOID)

WATER OTHER X Strong oxidizing or reducing agents.

***** VI - SPILL AND DISPOSAL INFORMATION *****

STEPS TO BE TAKEN IN CASE MATERIAL IS RELEASED OR SPILLED

Warning! Dusts may be explosive. If wet, floors may become slippery. Wear appropriate protective gear and respiratory protection where particulates of unknown concentrations may be generated (self-contained breathing apparatus preferred for large spills).

Carefully shovel spills (avoid generating dust) into appropriate containers for disposal. To remove residue, wet with water, absorb with sand or vermiculite and place in compatible container for disposal. Keep out of sewers and open bodies of water.

WASTE DISPOSAL METHODS

Dispose of in compliance with all Federal, state and local laws and regulations. Incineration is the preferred method.

CONTAINER DISPOSAL

Completely empty liner by shaking and tapping sides and bottom to loosen clinging particles. Empty residue into application equipment. Then dispose of liner in a sanitary landfill or by incineration if allowed by State and local authorities. If drum is contaminated and cannot be reused, dispose of in the same manner.

***** VII - PERSONAL PROTECTION INFORMATION *****

VENTILATION TYPE

In processes where dusts may be generated, proper ventilation must be provided in accordance with good ventilation practices.

RESPIRATORY PROTECTION

A NIOSH/MSHA jointly approved respirator is advised in the absence of proper environmental controls or if recommended TWA/TLV is exceeded.

PROTECTIVE GLOVES

Rubber or neoprene, when needed, to prevent skin contact.

EYE PROTECTION

Wear chemical goggles where there is a potential for eye contact. Use safety glasses with side shields under normal use conditions.

OTHER PROTECTIVE EQUIPMENT

Eye wash; safety shower; protective clothing (long sleeves, coveralls or other, as appropriate), when needed, to prevent skin contact.

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EMERGENCY TELEPHONE
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HEALTH	3
FLAMMABILITY	1
REACTIVITY	0

5970 Hyamine 1622 Crystals

PAGE 4 OF 6

***** VIII - STORAGE AND HANDLING *****

PRECAUTIONS FOR STORAGE AND HANDLING:

Maximum storage temperature: 140°F.

Do not contaminate drinking water, food or feed by storage or disposal.

Keep containers closed until used.

***** IX - TOXICOLOGY INFORMATION *****

- oral LD₅₀ (rat): 420 mg/kg (based on 100% active) (Lonza data)
- dermal LD₅₀ (rabbit): 3000 mg/kg (Lonza data)
- in rabbits, 100% active on skin was a severe irritant (Lonza data)
- in rabbits, daily dermal application of 0.1%, five days/week for 4 weeks did not produce systemic effects (CITFA Review)
- in rabbits, instillation to eye (50% active) produced irritation (Lonza data)
- in a teratology study, rats given up to 35.6 mg/kg/day on day 6 to 15 gestation resulted in maternal toxicity at the highest dose (CITFA Review)
- not mutagenic in various strains of Salmonella with or without metabolic activation (CITFA Review)
- not carcinogenic in rats when administered in diet at levels of 50 to 5000 ppm for two years (CITFA Review)
- in humans, a 5% aqueous solution, applied for 48 hours under occlusion caused irritation characterized by redness (CITFA Review)
- in humans, 0.1 to 0.2% as a tincture, aqueous solution or aerosol, was non-irritating (CITFA Review)
- in humans, three cases of topical sensitization reported in individuals using personal care products containing this compound (CITFA Review)

***** X - MISCELLANEOUS AND REGULATORY INFORMATION *****

NOTE TO PHYSICIAN:

Acute effects may include mucosal damage, severe laryngeal edema and shock associated with corrosive agents. Alcohol can increase toxic effects. Delayed effects may include life threatening respiratory paralysis and convulsions.

FEDERAL LEVEL REGULATIONS:

This is an EPA registered pesticide (EPA Registration No. 6836-91).

This material can only be used commercially in the EPA registered application(s) noted on the product label.

TOXIC SUBSTANCES CONTROL ACT (TSCA INVENTORY) STATUS:

Found on the U.S. EPA TSCA inventory.

MATERIAL SAFETY DATA SHEET

LONZA

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 800-424-9300 (CHEMTREC)

HEALTH	3
FLAMMABILITY	1
REACTIVITY	0

5970 Hyamine 1622 Crystals

PAGE 5 OF 6

***** X - MISCELLANEOUS AND REGULATORY INFORMATION (continued) *****

FEDERAL LEVEL REGULATIONS (continued):

CERCLA (Comprehensive Environmental Response, Compensation and Liability Act of 1980 requires notification of the National Response Center (Telephone 1 -800-424-8802) in the event of a release of quantities of the following hazardous materials contained in this product, if the release is equal to or greater than the Reportable Quantities (RQs) listed in 40 CFR 302.4:

<u>Chemical Name</u>	<u>CAS Number</u>	<u>Typical Maximum Concentration</u>
None known		

SARA Title III, Sections 302/304 (Superfund Amendments and Reauthorization act of 1986) - This act requires emergency planning, including agency notification, for possible release of the following components of this material, based upon the Threshold Planning Quantities (TPQs) and release Reportable Quantities (RQs) listed for the components in 40 CFR 355:

<u>Chemical Name</u>	<u>CAS Number</u>	<u>Typical Maximum Concentration</u>
None known		

SARA Title III Sections 311/312 - This act requires reporting under the Community Right-to-Know provisions due to the inclusion of the following components of this material in one or more of the five hazard categories listed in 40 CFR 370:

<u>Chemical Name</u>	<u>CAS Number</u>	<u>Hazard *) Categories</u>
Diisobutylphenoxyethoxyethyl-dimethylbenzyl-ammonium chloride monohydrate	121-54-0	A

*) The five hazard categories are as follows: F= FIRE HAZARD; S= SUDDEN RELEASE OF PRESSURE; R= REACTIVE; A= IMMEDIATE (ACUTE) HEALTH HAZARD; C= DELAYED (CHRONIC) HEALTH HAZARD

SARA Title 313 - This act requires submission of annual reports of releases of the following components of this material if the threshold reporting quantities, as listed in 40 CFR 372, are met or exceeded:

<u>Chemical Name</u>	<u>CAS Number</u>	<u>Typical Maximum Concentration</u>
Mono- di- and triethylene glycol ethers	Not applicable	100%

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EMERGENCY TELEPHONE
800-424-9300 (CHEMTREC)

HEALTH 3
FLAMMABILITY 1
REACTIVITY 0

5970 Hyamine 1622 Crystals

PAGE 6 OF 6

***** X - MISCELLANEOUS AND REGULATORY INFORMATION (continued) *****

STATE RIGHT-TO-KNOW REGULATIONS:

CALIFORNIA PROPOSITION 65 - Components present in this material which the State of California has found to cause cancer, birth defects or other reproductive harm are as follows:

<u>Chemical Name</u>	<u>CAS Number</u>	<u>Typical Maximum Concentration</u>
Benzene	71-43-2	2 ppm
Benzyl chloride	100-44-7	10 ppm
Bis(2-chloroethyl) ether	111-44-4	10 ppm
N-Nitrosodiethanolamine	1116-54-7	1 ppm
Propylene oxide	75-56-9	10 ppm
Toluene	108-88-3	Trace

MASSACHUSETTS Right-to-Know - The following components of this material are included in the Massachusetts Substance List and are present at or above reportable levels:

<u>Chemical Name</u>	<u>CAS Number</u>	<u>Typical Maximum Concentration</u>
Benzene	71-43-2	2 ppm
Benzyl chloride	100-44-7	10 ppm
Bis(2-chloroethyl) ether	111-44-4	10 ppm
N-Nitrosodiethanolamine	1116-54-7	1 ppm
Propylene oxide	75-56-9	10 ppm

MICHIGAN Critical Materials - The following components of this material are included in the Michigan Critical Materials List:

<u>Chemical Name</u>	<u>CAS Number</u>
None known	

NEW JERSEY Right-to-Know - The following components of this material are included in the New Jersey Hazardous Substance List and are present at or above reportable levels:

<u>Chemical Name</u>	<u>CAS Number</u>	<u>Typical Maximum Concentration</u>
None known		

PENNSYLVANIA Right-to-Know - The following components of this material are included in the Pennsylvania Hazardous Substance List and are present at or above reportable levels:

<u>Chemical Name</u>	<u>CAS Number</u>	<u>Typical Maximum Concentration</u>
None known		

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ATTACHMENT 3
TEST SUBSTANCE PREPARATION PROCEDURE

ATTACHMENT 3

Protocol 720-002
Version: 720-002 (10 APR 95)
Page 1 of 2

TEST ARTICLE/SUBSTANCE PREPARATION PROCEDURE

TA/S: Hyamine® 1622 (Chemical Name: Benzethonium Chloride)Vehicle: Reverse Osmosis Processed Deionized Water (R.O. deionized water)

A. Purpose:

The purpose of this procedure is to provide a method for the preparation of dosage solutions of Hyamine® 1622 for oral administration to rats on Argus Study 720-002.

B. General Information:

1. All solution containers will be labeled and color-coded (white, yellow, blue and red for each dosage group I through IV, respectively). Each label will specify the protocol number, TA/S identification, Argus batch number, concentration, dosage level, preparation date, expiration date and storage conditions.
2. Solutions will be prepared:

<input type="checkbox"/>	Daily	<input type="checkbox"/>	Weekly	<input checked="" type="checkbox"/>	For <u>21</u> days of use
<input type="checkbox"/>	As required by Protocol			<input type="checkbox"/>	By Sponsor
3. Solutions will be prepared at a final dosage volume of 10 mL/kg.
4. Safety:

<input checked="" type="checkbox"/>	Gloves, lab coat, goggles or safety glasses and faceshield
<input checked="" type="checkbox"/>	Dust-Mist Respirator
<input type="checkbox"/>	Half-Face Respirator
<input type="checkbox"/>	Full-Face Respirator/Positive Pressure Hood
<input type="checkbox"/>	Tyvek Suit/Apron
5. Dosage solutions adjusted for % Activity/Purity or Correction Factor:

<input type="checkbox"/>	Yes	<input checked="" type="checkbox"/>	No (Calculations based on 100%)		
<input type="checkbox"/>	% Activity	<input type="checkbox"/>	% Purity	<input type="checkbox"/>	Correction Factor
6. Sampling requirements: Cited in protocol
7. Storage: Cited in protocol

ATTACHMENT 3

Protocol 720-002
Version: 720-002 (10 APR 95)
Page 2 of 2

TEST ARTICLE/SUBSTANCE PREPARATION PROCEDURE

C. Dosage Solution Preparation:

1. For each liter of solution required, transfer the required amount of the Hyamine® 1622 into an appropriate container at least 1200 mL in size.
2. Slowly add approximately 800 mL of R.O. deionized water to the container, swirling to mix.
3. Insert a magnetic stir bar (about 5 cm in length) into the container and place on a magnetic stir plate, stir to achieve good mixing with minimum cavitation or bubble formation; stir until clear.
4. When the test substance appears to have dissolved, pour the contents into a 1 liter volumetric flask. Rinse the container at least once with R.O. deionized water and add each rinse to the volumetric flask. Then add additional R.O. deionized water to obtain a final volume of 1 liter.
5. Pour the contents of the volumetric flask into an appropriate container with a magnetic stirbar; place on a magnetic stir plate and continue stirring until samples are taken.
6. Repeat steps (1) through (5) for each concentration.

Written By: Mark A. CohenApproved by: John L. Foss Date: 18 May 95Modification:
☐ No ☒ Yes [see attached modification form]Initials/Date: MC 6-21-95

ARGUS

TEST ARTICLE/SUBSTANCE PREPARATION
PROCEDURE MODIFICATIONProtocol: 720-002Version: 720-002 (10.APR.95)

Date of Modification	Preparation Step #	Modification
<u>5-24-95</u>	<u>B.1.</u>	<u>1st sentence should read: 'All solution</u> <u>containers will be labeled and color-coded</u> <u>(white, yellow, green, blue and red for</u> <u>each dosage group I through V, respectively).</u> <u>Jim G. [Signature]</u> <u>25 May 95</u> <u>Study Director</u> <u>Date</u>
_____	_____	_____ _____ _____ _____ _____ _____ <u>Study Director</u> <u>Date</u>
_____	_____	_____ _____ _____ _____ _____ _____ <u>Study Director</u> <u>Date</u>
_____	_____	_____ _____ _____ _____ _____ _____ <u>Study Director</u> <u>Date</u>

Reviewed by: Jim G. [Signature]Date: 25 May 95



Argus Research Laboratories, Inc.
905 Sheehy Drive, Building A
Horsham, Pennsylvania 19044
T: (215) 443-8710 F: (215) 443-8587

PROTOCOL 720-002

DEVELOPMENTAL TOXICITY STUDY OF HYAMINE® 1622 IN RATS

Amendment 1 - October 23, 1995

1. Test Substance (page 3 of the protocol):

On October 10, 1995, a 10 g sample of the bulk test substance was sent at ambient temperature to the Sponsor for a post-study storage stability analysis. The sample was sent to:

Mr. Robert J. Sloan
Senior Research Chemist
Lonza Inc. (R&D)
79 Route 22 East
P.O. Box 993
Annandale, NJ 08801

The results of these analyses will be included in the raw data.

Reason for Change:

This change was made at the request of the Sponsor's Representative in order to confirm the purity of the test substance after storage at the Testing Facility.

Mildred S. Christian 10/23/95
Mildred S. Christian, Ph.D., ATS Date
Executive Director of Research

Alan M. Hoberman 10/23/95
Alan M. Hoberman, Ph.D., DABT Date
Director of Research

John A. Foss 23 October 95
John A. Foss, Ph.D. Date
Group Leader and Study Director

Michael W. Gill 10/24/95
Michael W. Gill, Ph.D. Date
Sponsor's Representative

APPENDIX 2

DEVIATIONS FROM THE PROTOCOL AND THE STANDARD OPERATING PROCEDURES OF THE TESTING FACILITY

DEVIATIONS FROM THE PROTOCOL AND THE STANDARD OPERATING PROCEDURES OF THE TESTING FACILITY

1. Chlorine levels in the Testing Facility water supply were not analyzed for the months of May and June, 1995. This oversight occurred as a result of failure of the contractor to analyze for chlorine content when the water sample was taken at the Testing Facility. This deviation had no effect on the outcome of the study because weekly chlorine analyses, during these months, conducted by Testing Facility personnel indicated that chlorine levels were within laboratory standards.
2. Feed consumption values were not recorded on day 15 of presumed gestation (June 7, 1995), for the following rats: 6801 - 6809 (Group I), 6827 - 6834 (Group II), 6851 - 6861 (Group III), 6876 - 6887 (Group IV) and 6901 - 6909 (Group V). This deviation did not adversely affect the outcome or interpretation of the study because feed values for these rats were recorded on day 16 of presumed gestation, which was the endpoint required for feed consumption for the entire dosage period; the day 15 of presumed gestation feed value was not essential for the evaluation of test substance effects.
3. At the time of necropsy on day 20 of presumed gestation (June 12, 1995), the maternal gross finding for Group II rat 6829, "localized alopecia, limbs 58, 59 - 1.0 x 0.5 cm", was inadvertently not confirmed by a laboratory supervisor. This deviation did not adversely affect the outcome or interpretation of the study because: 1) it was a single event; and 2) the observation is a common event in studies of this kind, and the incidence of alopecia in this location was unrelated to the test substance.
4. A uterine weight was inadvertently not recorded for Group III rat 6860 at necropsy on day 20 of presumed gestation (June 12, 1995). This deviation did not adversely affect the outcome or interpretation of the study because: 1) it was a single event; and 2) the remaining sample size was sufficient to detect effects of the test substance.

5. Group I fetus 6817-10 was examined for visceral alterations and sex. These data were inadvertently not entered at the time of examination. Although the sex of the fetus was noted on the tag for this fetus, there is no record whether or not the fetus appeared normal in the visceral examination. This deviation did not adversely affect the outcome or interpretation of the study because it was a single event. This deviation is noted in the summary and individual fetal alterations tables.

All deviations are documented in the raw data.

John A. Foss 26 Oct 95
John A. Foss, Ph.D. Date
Group Leader and Study Director

APPENDIX 3
ANALYTICAL REPORT

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Abstract:

The following investigation was conducted to provide analytical support for Argus Research Laboratories, Inc. Study #720-002. The concentration of the test substance (Hyamine® 1622) in reverse osmosis deionized water was determined using high pressure liquid chromatography. The solution concentrations of Hyamine® 1622 used for the study were 0, 1.0, 3.0, 10.0, and 17.0 mg/mL. Following the development and validation of the analytical method, homogeneity, and stability were evaluated on trial preparations of solutions that bracketed at the high and low concentrations used in the study. In addition, concentration analyses were performed on the dosing solutions prepared for use in the study. A detailed description of the procedures used to prepare and analyze these samples is included in the attachments to this analytical report. The results of the analyses of the samples submitted for homogeneity and stability indicated that Hyamine® 1622 was homogeneously distributed in the dosing solutions and was stable for a period of at least 21 days. In addition, the concentrations of Hyamine® 1622 in aliquots of the four Hyamine® 1622 dosing solutions prepared for use in the study were within 7% of nominal. No test substance was detected in the dose solution prepared for the vehicle control group.

Method and Results:**Homogeneity:**

Homogeneity was evaluated for test substance solutions prepared at 0.3 and 20.0 mg/mL concentrations to ensure that the test substance was uniformly distributed throughout the formulations. These concentrations were selected because they bracketed the concentrations administered to the animals in the study. Two or three samples were analyzed from three regions of each solution (top, middle, and bottom). The results of these analyses are presented in Table 1.

The average and range of the test substance concentrations in the 0.3 and 20.0 mg/mL formulations were 0.30 mg/g (0.28 to 0.31 mg/g) and 20.5 mg/g (20.4 to 20.6 mg/g), respectively, indicating that the test substance was uniformly distributed throughout the formulations.

Stability and Longer-Term Homogeneity:

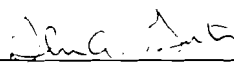
Stability and longer-term homogeneity was evaluated for the formulations prepared for the initial homogeneity analyses to ensure that the test substance solutions were stable and remained uniform during storage at Argus Research Laboratories. Two samples were analyzed from three regions of each formulation (top, middle, and bottom) after 14 and 21 days of storage at room temperature. The mean concentrations for each formulation determined at the time of the initial homogeneity analyses were used as the Day 0 reference values for the stability analyses. The results of these analyses are presented in Table 2 (Day 14 stability) and Table 3 (Day 21 stability).

The measured concentration for the 0.3 and 20.0 mg/mL solutions after 14 and 21 days of storage ranged from 100% to 107% of target for the 0.3 mg/mL solution and 98% to 102% of target for the 20 mg/mL solution. These results indicated that the test substance remained stable and uniformly distributed in the formulations during the 21-day period of storage.

Concentration Verification:

Samples of the dosing solutions prepared for administration to the animals in the study were analyzed by Lancaster Laboratories to confirm the concentration of the test substance. The dosing solutions were prepared once and analyzed prior to administration to the animals. The results of these analyses are presented in Table 4.

The measured concentration for all four dosage levels ranged from 101% to 107% of nominal. The test substance was not detected in any of the control dosing solutions.

Approved by: 
Name

Date: 10/25/95

Title: Gregory Heath

Analytical Report Table 1
Homogeneity

Lancaster Laboratories Sample #	Source (a)	Argus Sample Designation	Target Level mg/mL	Analytical Result mg/g	% Deviation from Target
2295315	Top	(04.12.95)-T	0.3	0.30	0
2295316	Top	(04.12.95)-T	0.3	0.30	0
2295317	Top	(04.12.95)-T	0.3	0.30	0
2295318	Middle	(04.12.95)-M	0.3	0.31	3
2295319	Middle	(04.12.95)-M	0.3	0.30	0
2295320	Middle	(04.12.95)-M	0.3	0.28	-6
2295321	Bottom	(04.12.95)-B	0.3	0.30	0
2295322	Bottom	(04.12.95)-B	0.3	0.30	0
2295323	Bottom	(04.12.95)-B	0.3	0.30	0
Avg:				0.30	0
RSD:				2.6%	
2308653	Top	(05.09.95)-T	20	20.4	2
2308654	Top	(05.09.95)-T	20	20.5	3
2308655	Middle	(05.09.95)-M	20	20.4	2
2308656	Middle	(05.09.95)-M	20	20.5	3
2308657	Bottom	(05.09.95)-B	20	20.6	3
2308658	Bottom	(05.09.95)-B	20	20.5	3
Avg:				20.5	3
RSD:				0.4%	

RSD = Relative standard deviation $[(\text{Standard Deviation} / \text{Avg}) * 100]$

Avg = Average

(a) Samples designated as originating from the top, middle, and bottom of the dose solution storage container.

Analytical Report Table 2
Stability and Longer-Term Homogeneity (Day 14)

Lancaster Laboratories Sample #	<u>Source (a)</u>	<u>Argus Sample Designation</u>	<u>Storage Time Days</u>	<u>Storage Condition</u>	<u>Analytical Result mg/g</u>	<u>% Deviation from Target (b)</u>
2302503	Top	(04.12.95)-T	14	Room Temp	0.30	0
2302504	Top	(04.12.95)-T	14	Room Temp	0.31	3
2302505	Middle	(04.12.95)-M	14	Room Temp	0.30	0
2302506	Middle	(04.12.95)-M	14	Room Temp	0.31	3
2302507	Bottom	(04.12.95)-B	14	Room Temp	0.31	3
2302508	Bottom	(04.12.95)-B	14	Room Temp	0.32	7
				Avg:	0.31	3
				RSD:	2.4%	
2317791	Top	(05.09.95)-T	14	Room Temp	20.6	0
2317792	Top	(05.09.95)-T	14	Room Temp	20.6	0
2317793	Middle	(05.09.95)-M	14	Room Temp	20.8	1
2317794	Middle	(05.09.95)-M	14	Room Temp	20.9	2
2317795	Bottom	(05.09.95)-B	14	Room Temp	20.4	0
2317796	Bottom	(05.09.95)-B	14	Room Temp	20.7	1
				Avg:	20.7	1
				RSD:	0.8%	

RSD = Relative standard deviation $[(\text{Standard Deviation} / \text{Avg}) * 100]$

Avg = Average

(a) Samples designated as originating from the top, middle, and bottom of the dose solution storage container.

(b) Targets were the averages of the Day 0 analyses: 0.30 mg/g and 20.5 mg/g (see Table 1).

Analytical Report Table 3
Stability and Longer-Term Homogeneity (Day 21)

Lancaster Laboratories Sample #	Source (a)	Argus Sample Designation	Storage Time Days	Storage Condition	Analytical Result mg/g	% Deviation from Target (b)
2306194	Top	(04.12.95)-T	21	Room Temp	0.30	0
2306195	Top	(04.12.95)-T	21	Room Temp	0.31	3
2306196	Middle	(04.12.95)-M	21	Room Temp	0.30	0
2306197	Middle	(04.12.95)-M	21	Room Temp	0.31	3
2306198	Bottom	(04.12.95)-B	21	Room Temp	0.31	3
2306199	Bottom	(04.12.95)-B	21	Room Temp	0.31	3
				Avg:	0.31	3
				RSD:	1.7%	
2320627	Top	(05.09.95)-T	21	Room Temp	20.3	-1
2320628	Top	(05.09.95)-T	21	Room Temp	20.1	-2
2320629	Middle	(05.09.95)-M	21	Room Temp	20.3	-1
2320630	Middle	(05.09.95)-M	21	Room Temp	20.4	0
2320631	Bottom	(05.09.95)-B	21	Room Temp	20.2	-1
2320632	Bottom	(05.09.95)-B	21	Room Temp	20.3	-1
				Avg:	20.3	-1
				RSD:	0.5%	

RSD = Relative standard deviation $[(\text{Standard Deviation} / \text{Avg}) * 100]$

Avg = Average

(a) Samples designated as originating from the top, middle, and bottom of the dose solution storage container.

(b) Targets were the averages of the Day 0 analyses: 0.30 mg/g and 20.5 mg/g (see Table 1).

Analytical Report Table 4
Concentration Verification

Lancaster Laboratories <u>Sample #</u>	Sample Preparation <u>Date</u>	Argus Sample <u>Designation</u>	Target Level <u>mg/mL</u>	Analytical Result <u>mg/g</u>	% Deviation <u>from Target</u>
2317781	5/23/95	A-(05.23.95)	0.0	<0.005	-----
2317782	5/23/95	A-(05.23.95)	0.0	<0.005	-----
2317783	5/23/95	B-(05.23.95)	1.0	1.07	7
2317784	5/23/95	B-(05.23.95)	1.0	1.07	7
			Avg:	1.07	7
2317785	5/23/95	C-(05.23.95)	3.0	3.05	2
2317786	5/23/95	C-(05.23.95)	3.0	3.05	2
			Avg:	3.05	2
2317787	5/23/95	D-(05.23.95)	10.0	10.1	1
2317788	5/23/95	D-(05.23.95)	10.0	10.2	2
			Avg:	10.2	2
2317789	5/23/95	E-(05.23.95)	17.0	17.5	3
2317790	5/23/95	E-(05.23.95)	17.0	17.5	3
			Avg:	17.5	3



Analysis #NS-08-125
Initiated Date: 03/31/95
Effective Date: APR 10 1995

Appendix I
Analytical Procedure
Hyamine 1622
in R.O. Deionized Water

Reference:

All analytical work was conducted by Lancaster Laboratories, Inc.

Scope:

Applicable to the determination of Hyamine 1622 in reverse osmosis (R.O.) deionized water.

Basic Principles:

The analytical procedure consists of transferring the sample into a volumetric flask, diluting the sample with mobile phase, diluting aliquots to the appropriate concentrations in the solution, and analyzing by HPLC.

Safety Precautions:

Follow all general laboratory procedures. Take general precautions when handling Hyamine 1622 standard. Wear gloves and safety glasses and avoid inhalation of the powder.

Apparatus and Reagents:

1. Shimadzu LC-600 pumping system or equivalent
2. Shimadzu SPD-6A UV absorbance detector or equivalent
3. Shimadzu SCL-6A controller or equivalent
4. Chrom Perfect data system or equivalent
5. Waters uBondapack C-18 3.6 mm x 30 cm or equivalent
6. Routine laboratory equipment and glassware

Analysis #NS-08-125
 Initiated Date: 03/31/95
 Effective Date: APR 10 1995
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7. Methanol, HPLC grade or equivalent
8. Phosphoric acid, ACS grade or equivalent
9. 50 mM KH_2PO_4 - Weigh approximately 6.8 g KH_2PO_4 into a beaker. Add 1 liter R.O. deionized water.
10. Mobile phase - Prepare a solution of 80% Methanol/20% 50 mM KH_2PO_4 , adjust to pH 3.0 with phosphoric acid and vacuum filter through a membrane.
11. Hyamine 1622. analytical standard - Lot #40109, purity 99.3% supplied by the sponsor. Store at room temperature.
12. Stock standard - Accurately weigh approximately 100 mg to the nearest 0.1 mg of Hyamine 1622 standard into a 100-ml volumetric flask. Dissolve in and dilute to volume with mobile phase. The necessity of preparing a fresh standard is dependent upon the standard stability in solution. See Appendix II for this data. The client supplied Hyamine 1622 standard lot #40109, 99.3% purity.
13. Working standard solutions - Dilute aliquots of the stock standard to achieve the following recommended concentrations: 0.6, 0.2, 0.1, 0.06, and 0.01 mg/ml.
14. Spike preparation - Spikes should be prepared to simulate the lowest and highest sample concentrations in the analysis. Spiking solutions may be pipetted or standard weighed and added to the appropriate weight of vehicle.

Procedure:

A. Sample preparation

1. Accurately weigh the bottle, cap, and sample; record the weight. Quantitatively transfer the entire sample with mobile phase to appropriate volumetric flasks. Allow the bottle and cap to dry, weigh, record the weight, and determine the sample weight by difference.

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 Initiated Date: 03/31/95
 Effective Date: APR 10 1995
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2. Dissolve and dilute the sample to volume with mobile phase.
3. Make appropriate final dilutions with mobile phase to obtain a final solution of approximately 0.2 mg/ml.

B. HPLC determination

1. Set the HPLC as described in the Apparatus section to the following conditions:

Detector wavelength	220 nm
Injection volume	20 μ l
Flow rate	1 ml/min

(These conditions may be changed for optimum integration.)

2. After the column has equilibrated, make injections of a standard until peak heights and retention times are consistent.
3. Inject 20 μ l of each sample and standard. A standard curve solution should be run after every four sample injections.

Calculations:

1. Calibration may be performed prior to running the samples, while the samples are run, or after all samples are run. Any curve may be used providing standard continuity is maintained throughout the run. Determine the linear fit for the curve with the Chrom Perfect software to calibrate.
2. The correlation coefficient must be greater than or equal to 0.995.
3. Calculate the Hyamine 1622 concentrations in the samples using this calibration.
4. At least one injection of the high-level standard and one of the middle-level standard should be included within the analytical run. When calculated against the calibration curve, these standards should not deviate from the nominal

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Initiated Date: 03/31/95
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concentration by more than $\pm 5\%$. If the standard is outside of this range, consult SOP-FC-002, "Statistical Evaluation of Chromatographic Data Used for Support of Toxicology Studies."

NOTE: All sample responses must fall within the standard curve.

Quality Assurance:

1. A standard should be run after every fourth sample injection.
2. Run at least five different concentrations of the stock standard for a standard curve.
3. Spike recovery should be performed at both the highest and lowest target concentrations of Hyamine 1622 in the samples. If only one level is being tested, one spike prepared to simulate the level is necessary.

NS08125.W60
040695

Prepared by: *Mark A. Smith* Date: 4/7/95

Title: CHEMIST II Date: 4/7/95

Approved by: *Alan G. Hunt* Date: 4/7/95

Title: Group Leader Date: 4/7/95

Approved by: *Dan G. Vanden* Date: 4/7/95

Title: QUALITY ASSURANCE SPECIALIST Date: 4/7/95



Analytical Procedure Validation
for Analysis #NS-08-125
Initiated Date: 03/31/95
Effective Date:

APR 10 1995

Appendix II
Analytical Procedure Validation
Hyamine 1622 in
R.O. Deionized Water

The analytical method was validated for the determination of Hyamine 1622 in R.O. deionized water. Five samples each of the 10-mg/ml and 160-mg/ml levels were tested to ensure method precision. Spikes of R.O. deionized water (0 mg/ml) were prepared and analyzed to demonstrate recovery. Linearity and a stability check of standard solutions were performed to demonstrate adequacy of the method. The method validation consists of the following:

Method:

1. Blank preparation

A 2-ml aliquot of R.O. deionized water was taken and prepared to simulate the lowest level concentration (10 mg/ml) beginning with step A.(2) of the analytical procedure. The sample represents the greatest amount of R.O. deionized water found in the prepared samples and shows the absence of any interfering peaks.

2. Spike sample preparation

Spikes were prepared at the 10-mg/ml and 160-mg/ml levels. The 10-mg/ml samples were prepared by accurately weighing approximately 0.02 g Hyamine 1622 into a 100-ml volumetric flask, dissolving it in 2 ml R.O. deionized water, and diluting to volume with mobile phase.

The 160-mg/ml samples were prepared by accurately weighing approximately 0.32 g Hyamine 1622 into a 100-ml volumetric flask, dissolving it in 2 ml R.O. deionized water, and diluting to volume with mobile phase.

Analytical Procedure Validation
 for Analysis #NS-08-125
 Initiated Date: 03/31/95
 Effective Date: APR 10 1995
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System Suitability:

1. To ensure system suitability for the determination of Hyamine 1622 in the mobile phase, a resolution factor was determined from the HPLC chromatogram. A resolution factor of approximately 1.5 indicates complete resolution of the Hyamine 1622 peak from other peaks in the chromatogram. A resolution factor of >1.5 was observed on all chromatograms, which verifies baseline resolution between Hyamine 1622 and other components.
2. To ensure stability of Hyamine 1622 in the analysis solvent, five replicates of a standard solution stored at ambient temperature for 58 hours were analyzed against a freshly prepared standard solution. The calculated response factors were averaged for each standard and the ratio of the average response factor were compared as described in Lancaster Laboratories SOP-FC-001, "Validation of Chromatographic Methods Used for Support of GLP Toxicology Studies in Department 08." The results are as follows:

<u>Stored</u> <u>Standard Response</u> <u>Factors (X10⁻³)</u>	<u>Fresh</u> <u>Standard Response</u> <u>Factors (X10⁻³)</u>
4.1524	4.1975
4.1457	4.2065
4.1691	4.2242
4.1474	4.2132
4.1653	4.2568
Mean(Rf1) = 4.1560	Mean(Rf2) = 4.2196
RSD = 0.26	RSD = 0.54

$$\frac{Rf2}{Rf1} = \frac{4.2196}{4.1560} = 1.015 \text{ and } 0.98 \leq 1.015 \leq 1.02$$

The relative standard deviations of the response factors for both sets of data are less than or equal to $\pm 2.0\%$.

The evaluation of the data indicates that the areas are statistically similar. All analytical runs should fall within a 58-hour period.

Analytical Procedure Validation
 for Analysis #NS-08-125
 Initiated Date: 03/31/95
 Effective Date: APR 10 1995
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Recovery:

The recovery results for the spikes prepared with R.O. deionized water
 (0 mg/ml) are as follows:

<u>Date</u>	<u>Spike Concentrations (mg/ml)</u>	<u>Quantity Recovered</u>	<u>Percent Recovered</u>
03/19/95	11.60	11.6653	100.6%
03/19/95	10.45	10.4572	100.1%
03/19/95	10.70	10.8421	101.3%
03/19/95	9.75	9.9053	101.6%
03/19/95	10.90	11.0965	101.8%
RSD = 0.72%			
Mean = 101.1%			
03/19/95	172.25	179.1578	104.0%
03/19/95	168.35	176.2620	104.7%
03/19/95	163.00	163.7208	100.4%
03/19/95	162.00	167.7133	103.5%
03/19/95	166.65	171.4756	102.9%
RSD = 1.58%			
Mean = 103.1%			

Precision:

Precision was checked using five samples each of two concentration levels. These samples were analyzed by a Lancaster Laboratories, Inc. employee to validate precision. The following data indicates acceptable precision.

<u>Target Level (mg/ml)</u>	<u>Replicate Number</u>	<u>Quantity Found (mg/ml)</u>
10	1	10.2612
	2	10.1889
	3	10.3099
	4	10.1808
	5	10.2569
Mean = 10.24		
RSD = 0.53%		

Analytical Procedure Validation
 for Analysis #NS-08-125
 Initiated Date: 03/31/95
 Effective Date: APR 10 1995
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<u>Target Level (mg/ml)</u>	<u>Replicate Number</u>	<u>Quantity Found (mg/ml)</u>
160	1	159.3277
	2	164.7243
	3	161.2026
	4	159.5100
	5	159.0265

Mean = 160.8
 RSD = 1.48%

Linearity:

To ensure the accuracy of the results a linearity study of the detector response versus Hyamine 1622 concentrations was conducted. Solutions of Hyamine 1622 ranging in concentration from approximately 1 mg/ml to 0.001 mg/ml were chromatographed and plotted. The coefficient of determination of the standard calibration was 0.9999845 which indicates linearity of the method over the tested range. All Hyamine 1622 determinations were chromatographed at concentrations within this linear range.

Detection Limit:

The lowest concentration of Hyamine 1622 standard which could be detected, 0.001 mg/ml, describes the instrument detection limit. This standard was used to calculate the limit of detection, 0.05 mg/ml. Limit of detection describes samples that show no detectable signal and are below the region of less-certain quantitation. They are reported as "none detected" (ND) and the limit of detection is then given.

$$LOD = 0.001 \text{ mg/ml} \times \frac{100 \text{ ml}}{2 \text{ grams}} = 0.05 \text{ mg/ml}$$

Limit of quantitation (LOQ) is defined as the level above which quantitation results may be obtained with a specified degree of confidence. The LOQ can be calculated from the lowest standard concentration in the curve as follows:

Analytical Procedure Validation
for Analysis #NS-08-125
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$$\frac{0.01 \text{ mg/ml} \times 100}{2 \text{ grams}}$$

The LOQ for this method is 0.5 mg/ml.

NS08125.W60
040695

Prepared by: Mark A. Lutz Date: 4/7/95

Title: CHEMIST II Date: 4/7/95

Approved by: Alan A. Lutz Date: 4/7/95

Title: Group Leader Date: 4/7/95

Approved by: Dan G. Lutz Date: 4/7/95

Title: QUALITY ASSURANCE SPECIALIST Date: 4/7/95

Analytical Procedure Validation
for Analysis #NS-08-125
Initiated Date: 03/31/95
Effective Date: APR 10 1995
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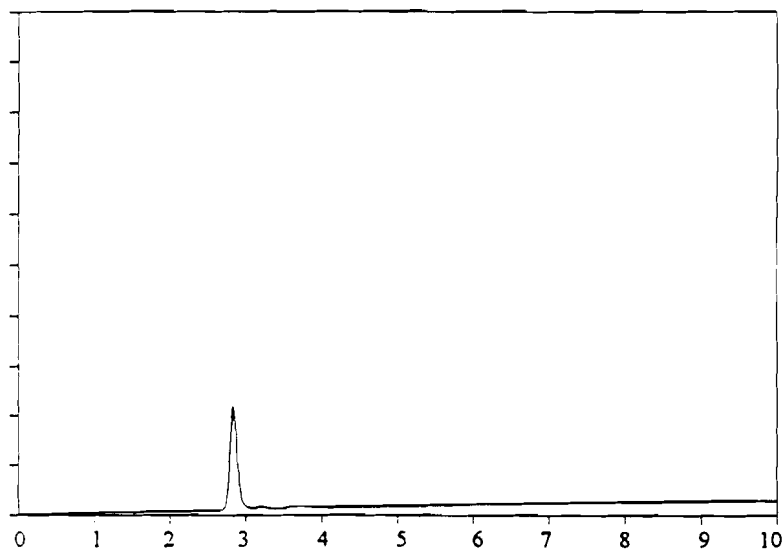


FIGURE I
Blank

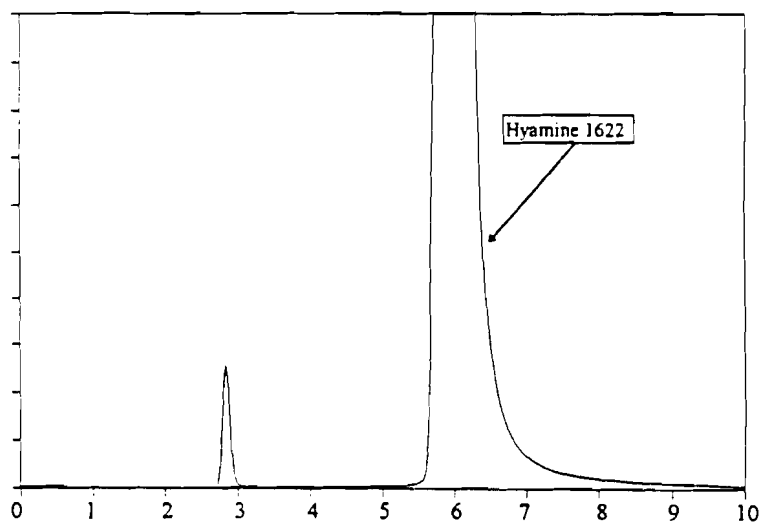


FIGURE II
Typical Sample Chromatogram

Analytical Procedure Validation
for Analysis #NS-08-125
Initiated Date: 03/31/95
Effective Date:
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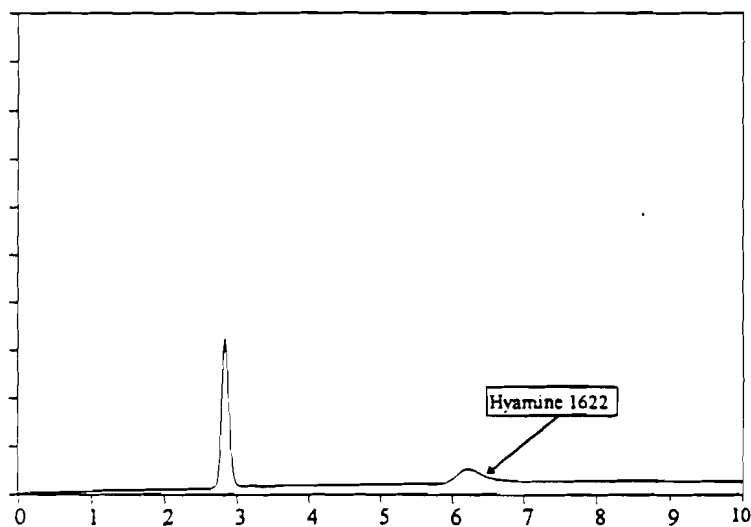


FIGURE III
Detection Limit Chromatogram

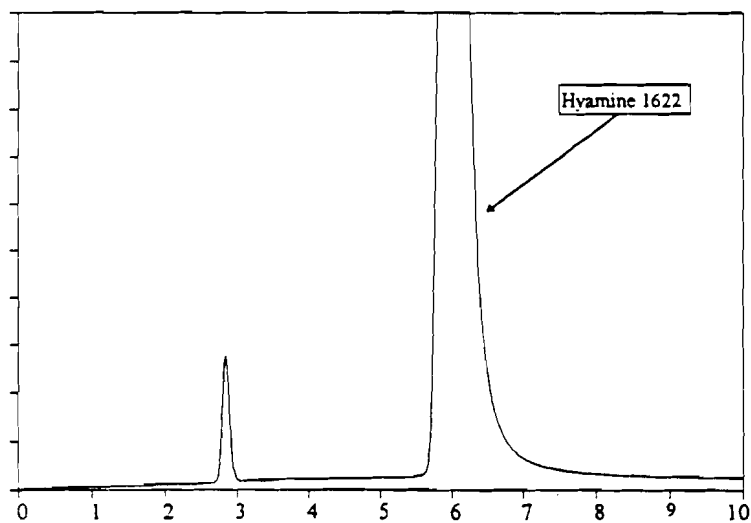


FIGURE IV
Typical Standard Chromatogram



Procedural Amendment #1

Number: NS-08-125

Title: Analytical Procedure Validation Hyamine 1622 in R.O. Deionized Water

Effective Date (listed on procedure): 04/10/95

Section(s) affected by change: Method, Recovery, Precision, Detection Limit

Reason for addition(s) or change(s): The analytical method was validated for the determination of Hyamine 1622 in R.O. deionized water for the concentration levels of 10 mg/ml and 160 mg/ml. The client submitted another level, 0.03 mg/ml. The calibration range is too large for quantitation at this low level. Using the previously validated linearity, the calibration range was shifted towards the lower concentrations. Five samples of the 0.03 mg/ml level were tested to ensure method precision. Spikes of R.O. deionized water (0 mg/ml) were prepared and analyzed to demonstrate recovery.

Change will be effective from (date): 04/18/95

Samples or project affected: 720-001, 720-002P

List change(s) or addition(s) (specify which section):

Method:

1. Blank preparation (change to the following)

A 2-ml aliquot of R.O. deionized water was taken and prepared to simulate the 0.03 mg/ml level concentration beginning with step 1.(a) of the analytical procedure. The sample represents the greatest amount of R.O. deionized water found in the prepared samples and shows the absence of any interfering peaks.

2. Spike sample preparation (add the following)

The 0.03 mg/ml samples were prepared by accurately weighing approximately 2 ml into a 10-ml volumetric flask and pipetting 1 ml of the approximate 0.06 mg/ml was pipetted into the flask. The flask was diluted to volume with mobile phase.

Recovery: (add the following)

<u>Date</u>	<u>Spike Concentrations (mg/ml)</u>	<u>Quantity Recovered (mg/ml)</u>	<u>Percent Recovered</u>
04/19/95	0.03028	0.0275	90.8%
04/19/95	0.03054	0.0287	94.0%
04/19/95	0.02996	0.0274	91.5%
04/19/95	0.03041	0.0277	91.1%
04/19/95	0.03023	0.0285	94.3%

RSD = 1.8%
Mean = 92.3%

Procedural Amendment #1
 Analytical Procedure Validation
 for Analysis #NS-08-125
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Precision: (add the following)

Target Level (mg/ml)	Replicate Number	Quantity Found (mg/ml)
0.03	1	0.0274
	2	0.0259
	3	0.0264
	4	0.0268
	5	0.0267

Mean = 0.02664
 RSD = 2.1%

Detection Limit: (change to the following)

The lowest concentration of Hyamine 1622 standard which could be detected, 0.001 mg/ml, describes the instrument detection limit. This standard was used to calculate the limit of detection, <0.005 mg/ml. Limit of detection describes samples that show no detectable signal and are below the region of less-certain quantitation. They are reported as "none detected" (ND) and the limit of detection is then given.

$$LOD = 0.001 \text{ mg/ml} \times \frac{10 \text{ ml}}{2 \text{ grams}} = 0.005 \text{ mg/ml}$$

Limit of quantitation (LOQ) is defined as the level above which quantitation results may be obtained with a specified degree of confidence. The LOQ can be calculated from the lowest standard concentration in the curve as follows:

$$\frac{0.01 \text{ mg/ml} \times 10}{2 \text{ grams}}$$

The LOQ for this method is 0.05 mg/ml.

NS08125.W60
 042495

Prepared by: *Michael D. Smith* Date: 4/24/95

Title: *Analyst II* Date: 4/26/95

Approved by: *John A. Smith* Date: 4/26/95

Title: *Group leader* Date: 4/26/95

Approved by: *Dan J. Smith* Date: 5-1-95

Title: *QA SPECIALIST* Date: 5-1-95

Appendix III

Quality Assurance Statement

Argus Protocol Number: 720-002

The final report and raw data for study 720-002 was compared and audited for accuracy by the Lancaster Laboratories' Quality Assurance Unit.

Christa D. Hill
Quality Assurance Specialist

October 25, 1995
Date

APPENDIX 4
FEED ANALYSES



PMI FEEDS, INC.
1401 S. BROADWAY
ST. LOUIS, MO 63104

314 784-1111
314 784-1112

TO RICHMOND, IN

CC DAN HOPKINS, 1W
RICHMOND, IN
DAN HOPKINS, 1W

LAB NO 421911 ENTERED 02/23/95 REPORTED 03/07/95 RHI

8002

CERTIFIED RODENT DIET MEAL
LOT NUMBER FEB 22 95 30

ASSAY	ANALYSIS	UNITS
PROTEIN (N X 6.25)	20.7	%
FAT (ACID HYDRO.)	6.20	%
FIBER (CRUDE)	4.42	%
ARSENIC	0.226	PPM
CADMIUM	0.126	PPM
CALCIUM	0.725	%
LEAD	0.259	PPM
MERCURY	LESS THAN 0.05	PPM
PHOSPHORUS	0.632	%
SELENIUM	0.239	PPM

ORGANOPHOSPHATE PEST

(PPM)	(PPM)
DIAZINON..... LESS THAN 0.02	PARATHION..... LESS THAN 0.02
DISULFOTON..... LESS THAN 0.02	THIMET..... LESS THAN 0.02
ETHION..... LESS THAN 0.02	THIODAN..... LESS THAN 0.02
MALATHION..... 0.05	TRITHION..... LESS THAN 0.02
METHYL PARATHION... LESS THAN 0.02	

PESTICIDE & PCB

(PPM)	(PPM)
ALDRIN..... LESS THAN 0.02	ENDRIN..... LESS THAN 0.02
ALPHA-BHC..... LESS THAN 0.02	HCB..... LESS THAN 0.02
BETA-BHC..... LESS THAN 0.02	HEPTACHLOR..... LESS THAN 0.02
DELTA-BHC..... LESS THAN 0.02	HEPTACHLOR EPOXIDE..... LESS THAN 0.02
CHLORDANE..... LESS THAN 0.02	LINDANE..... LESS THAN 0.02
DDE..... LESS THAN 0.02	METHOXYCHLOR..... LESS THAN 0.02

EXACT COPY
3/6/2095



PMI Feeds, Inc.
1401 S. Delmar St.
St. Louis, MO 63104

TEL: 314-762-2562
FAX: 314-762-2561

RT LAB NUMBER 421911

CERTIFIED RODENT DIET MEAL
LOT NUMBER FEB 22 95 30

PESTICIDE & PCB (CONTINUED)

DDT... (TOTAL).....	LESS THAN 0.02	MIREX.....	LESS THAN 0.02
DIELDRIN.....	LESS THAN 0.02	PCB.....	LESS THAN 0.15

AFLATOXIN

TOTAL: LESS THAN 5 PPB

FOR ADDITIONAL INFORMATION, PLEASE CONTACT:

- (1) FOR ASSAY METHODOLOGY - MICHAEL J. MURPHY 314-982-2562
- (2) FOR NUTRITIONAL INTERPRETATION-DR DAN HOPKINS 314-762-4876
- (3) ALL OTHER QUESTIONS-RICHMOND, IN., MANUFACTURING PLANT 317-962-9561

EXACT COPY
By 6 20 95



PMI Seeds, Inc.
1401 S. Hannibal Road
St. Louis, MO 63104

(314) 768-4100
(314) 768-4101

TO RICHMOND, IN CC DAN HOPKINS, 1W
RICHMOND, IN
DAN HOPKINS, 1W

LAB NO 428668 ENTERED 03/28/95 REPORTED 04/06/95 RMI 8002

CERTIFIED RODENT DIET MEAL
LOT NUMBER MAR 27 95 28

ASSAY	ANALYSIS	UNITS
PROTEIN (N X 6.25)	21.1	%
FAT (ACID HYDRO.)	6.00	%
FIBER (CRUDE)	4.36	%
ARSENIC	0.328	PPM
CADMIUM	0.123	PPM
CALCIUM	0.894	%
LEAD	0.275	PPM
MERCURY	LESS THAN 0.05	PPM
PHOSPHORUS	0.634	%
SELENIUM	0.231	PPM

EXACT COPY

BVL 6 20 95

ORGANOPHOSPHATE PEST

(PPM)	(PPM)
DIAZINON..... LESS THAN 0.02	PARATHION..... LESS THAN 0.02
DISULFOTON..... LESS THAN 0.02	THIMET..... LESS THAN 0.02
ETHION..... LESS THAN 0.02	THIODAN..... LESS THAN 0.02
MALATHION..... 0.05	TRITHION..... LESS THAN 0.02
METHYL PARATHION... LESS THAN 0.02	

PESTICIDE & PCB

(PPM)	(PPM)
ALDRIN..... LESS THAN 0.02	ENDRIN..... LESS THAN 0.02
ALPHA-BHC..... LESS THAN 0.02	HCB..... LESS THAN 0.02
BETA-BHC..... LESS THAN 0.02	HEPTACHLOR..... LESS THAN 0.02
DELTA-BHC..... LESS THAN 0.02	HEPTACHLOR EPOXIDE... LESS THAN 0.02
CHLORDANE..... LESS THAN 0.02	LINDANE..... LESS THAN 0.02
DDE..... LESS THAN 0.02	METHOXYCHLOR..... LESS THAN 0.02



PMI Feeds, Inc.
1401 S. Hannay Road
St. Louis, MO 63104

(314) 768-4460
(314) 768-4461

RT LAB NUMBER 428668

PAGE 2

CERTIFIED RODENT DIET MEAL
LOT NUMBER MAR 27 95 2B

PESTICIDE & PCB (CONTINUED)

DDT..(TOTAL).....	LESS THAN 0.02	MIREX.....	LESS THAN 0.02
DIELDRIN.....	LESS THAN 0.02	PCB.....	LESS THAN 0.18

AFLATOXIN

TOTAL: LESS THAN 5 PPB

FOR ADDITIONAL INFORMATION, PLEASE CONTACT:

- (1) FOR ASSAY METHODOLOGY - MICHAEL J. MURPHY 314-982-3562
- (2) FOR NUTRITIONAL INTERPRETATION-DR DAN HOPKINS 314-768-4576
- 3) ALL OTHER QUESTIONS-RICHMOND, IN., MANUFACTURING PLANT 317-962-9561

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JUL 6 20 95



PMI Feeds, Inc.
1401 S. Hanley Road
St. Louis, MO 63144

(314) 765-1100
(314) 765-4765 Fax

TO RICHMOND, IN

CC DAN HOPKINS, 1W
RICHMOND, IN
DAN HOPKINS, 1W

LAB NO 434870 ENTERED 05/02/95 REPORTED 05/12/95 RMI

5002

CERTIFIED RODENT DIET MEAL
LOT NUMBER APR 27 95 2B

ASSAY	ANALYSIS	UNITS
PROTEIN (N X 6.25)	21.0	%
FAT (ACID HYDRO.)	5.82	%
FIBER (CRUDE)	4.30	%
ARSENIC	0.272	PPM
RADIUM	0.140	PPM
CALCIUM	0.789	%
LEAD	0.251	PPM
MERCURY		

LESS THAN 0.05 PPM

SELENIUM 0.228 PPM

ORGANOPHOSPHATE PEST

	(PPM)		(PPM)
DIAZINON.....	LESS THAN 0.02	PARATHION.....	LESS THAN 0.02
DISULFOTON.....	LESS THAN 0.02	THIMET.....	LESS THAN 0.02
ETHION.....	LESS THAN 0.02	THIODAN.....	LESS THAN 0.02
MALATHION.....	0.05	TRITHION.....	LESS THAN 0.02
METHYL PARATHION...	LESS THAN 0.02		

PESTICIDE & PCB

	(PPM)		(PPM)
ALDRIN.....	LESS THAN 0.02	ENDRIN.....	LESS THAN 0.02
ALPHA-BHC.....	LESS THAN 0.02	PCB.....	LESS THAN 0.02
BETA-BHC.....	LESS THAN 0.02	HEPTACHLOR.....	LESS THAN 0.02
DELTA-BHC.....	LESS THAN 0.02	HEPTACHLOR EPOXIDE..	LESS THAN 0.02
CHLORDANE.....	LESS THAN 0.02	LINDANE.....	LESS THAN 0.02
DDE.....	LESS THAN 0.02	METHOXYCHLOR.....	LESS THAN 0.02
DOT..(TOTAL).....	LESS THAN 0.02	MIREX.....	LESS THAN 0.02
DIELDRIN.....	LESS THAN 0.02	PCB.....	LESS THAN 0.15

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By 6-20-95



PMI Feeds, Inc.
1401 S. Hanley Road
St. Louis, MO 63144

(314) 768-4100
(314) 768-4765 Fax

RT LAB NUMBER 434670

PAGE 2

CERTIFIED RODENT DIET MEAL
LOT NUMBER APR 27 95 2B

AFLATOXIN

TOTAL: LESS THAN 5 PPB

FOR ADDITIONAL INFORMATION, PLEASE CONTACT:

- (1) FOR ASSAY METHODOLOGY - MICHAEL J. MURPHY 314-982-3562
- (2) FOR NUTRITIONAL INTERPRETATION-DR DAN HOPKINS 314-768-4576
- (3) ALL OTHER QUESTIONS-RICHMOND, IN., MANUFACTURING PLANT 317-962-9561

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BY 6-20-95



PMI Feeds, Inc.
1401 S. Hanley Road
St. Louis, MO 63144

(314) 768-4100
(314) 768-4765 Fax

TO RICHMOND, IN

CC DAN HOPKINS, IW
RICHMOND, IN
DAN HOPKINS, IW

LAB NO 425239 ENTERED 05/03/95 REPORTED 05/15/95 RHI 5002

CERTIFIED RODENT DIET MEAL
LOT NUMBER APR 27 95 ZB

ASSAY	ANALYSIS	UNITS
PHOSPHORUS	0.897	%

FOR ADDITIONAL INFORMATION, PLEASE CONTACT:

- (1) FOR ASSAY METHODOLOGY - MICHAEL J. MURPHY 314-982-2562
- (2) FOR NUTRITIONAL INTERPRETATION-DR DAN HOPKINS 314-768-4576
- (3) ALL OTHER QUESTIONS-RICHMOND, IN., MANUFACTURING PLANT 317-962-9561

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BY 6 20 95



PMI FEEDS
100 S. BROADWAY
SILVER SPRING, MD 20910

501-221-1111
501-221-1112

TO RICHMOND, IN

CC DAN HOPKINS, 1W
RICHMOND, IN
DAN HOPKINS, 1W

LAB NO 434633 ENTERED 05/01/95 REPORTED 05/05/95 RHI

5002

CERTIFIED RODENT DIET MEAL
LOT NUMBER APR 27 95 2C

ASSAY	ANALYSIS	UNITS
PROTEIN (N X 6.25)	21.0	%
FAT (ACID HYDRO.)	5.96	%
FIBER (CRUDE)	4.38	%
ARSENIC	0.309	PPM
CADMIUM	0.139	PPM
CALCIUM	0.895	%
LEAD	0.274	PPM
MERCURY	LESS THAN 0.05	PPM
SELENIUM	0.246	PPM

EXACT COPY
Bx 6 20 95

ORGANOPHOSPHATE PEST

(PPM)	(PPM)
DIAZINON..... 0.06	PARATHION..... LESS THAN 0.02
DISULFOTON..... LESS THAN 0.02	THIMET..... LESS THAN 0.02
ETHION..... LESS THAN 0.02	THIODAN..... LESS THAN 0.02
MALATHION..... 0.05	TRITHION..... LESS THAN 0.02
METHYL PARATHION... LESS THAN 0.02	

PESTICIDE & PCB

(PPM)	(PPM)
ALDRIN..... LESS THAN 0.02	ENDRIN..... LESS THAN 0.02
ALPHA-BHC..... LESS THAN 0.02	MOB..... LESS THAN 0.02
BETA-BHC..... LESS THAN 0.02	HEPTACHLOR..... LESS THAN 0.02
DELTA-BHC..... LESS THAN 0.02	HEPTACHLOR EPOXIDE..... LESS THAN 0.02
CHLORDANE..... LESS THAN 0.02	LINDANE..... LESS THAN 0.02
DDE..... LESS THAN 0.02	METHOXYCHLOR..... LESS THAN 0.02
DDT (TOTAL)..... LESS THAN 0.02	MIREX..... LESS THAN 0.02
DIELDRIN..... LESS THAN 0.02	PCB..... LESS THAN 0.15



PMI FEEDS
1401 S. Highway 100
St. Louis, MO 63114

(314) 768-1000
(314) 768-1001

TO RICHMOND, IN

CC DAN HOPKINS, 1W
RICHMOND, IN
DAN HOPKINS, 1W

LAB NO 435238 ENTERED 05/03/95 REPORTED 05/15/95 RMI 5002

CERTIFIED RODENT DIET MEAL
LOT NUMBER APR 27 95 20

ASSAY	ANALYSIS	UNITS
PHOSPHORUS	0.586	%

FOR ADDITIONAL INFORMATION, PLEASE CONTACT:

- (1) FOR ASSAY METHODOLOGY - MICHAEL J. MURPHY 314-982-3562
- (2) FOR NUTRITIONAL INTERPRETATION-DR DAN HOPKINS 314-768-4576
- (3) ALL OTHER QUESTIONS-RICHMOND, IN., MANUFACTURING PLANT 317-962-9561

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3/6/20-95



PMI Feeds, Inc.
1401 S. Hixson Road
St. Louis, MO 63144

(314) 763-1100
(314) 763-1763 Fax
RT LAB NUMBER 434633

PAGE 2

CERTIFIED RODENT DIET MEAL
LOT NUMBER APR 27 95 20

AFLATOXIN

TOTAL: LESS THAN 5 PPB

FOR ADDITIONAL INFORMATION, PLEASE CONTACT:

- (1) FOR ASSAY METHODOLOGY - MICHAEL J. MURPHY 314-952-3562
- (2) FOR NUTRITIONAL INTERPRETATION-DR DAN HOPKINS 314-763-6576
- (3) ALL OTHER QUESTIONS-RICHMOND, IN., MANUFACTURING PLANT 317-962-9561

6-20-95

TOTAL P.03

APPENDIX 5
WATER ANALYSES

Weekly Chlorine Check
Room 14 (Animal Room)

Date	Concentration (ppm)
02 MAY 95	0.4
09 MAY 95	0.7
16 MAY 95	0.4
23 MAY 95	0.4
31 MAY 95	0.4
06 JUN 95	0.2
16 JUN 95	a

- a. Analysis for the chlorine content was not performed because the in-life portion of the study was completed.

Analysis Report**Lancaster Laboratories***Where quality is a science.*

Page: 1 of 4

LLI Sample No. WW 2250404

Collected: 1/18/95 at 08:50 by EA

Submitted: 1/18/95 Reported: 1/31/95

Discard: 3/ 3/95

905 Sheehy Formulation Laboratory Grab Water

Account No: 02423

Argus Research Labs., Inc.

935 Horsham Road

Horsham PA 19044-1230

P.O.

Rel.

John F. Smith
2/2/95

CAT NO.	ANALYSIS NAME	AS RECEIVED		
		RESULTS	LIMIT OF QUANTITATION	UNITS
0178	Pesticides/PCB's			See Page 2
1856	Method 8150 Herbicides			See Page 3
0514	Anion Scan			See Page 4

EXACT COPY

By 4-28-95

1 COPY TO Argus Research Labs., Inc. ATTN: Alan M Hoberman, Ph.D

Questions? Contact your Client Services Representative
 Jeannie Jacobson at (717) 656-2300
 04:11:14 D 0001 1 450795
 249 0.00 00061000 ASR000

Respectfully Submitted
 Jenifer E. Hess, B.S.
 Group Leader Pesticides/PCBs



Lancaster Laboratories Inc.
 2425 New Holland Pike
 Lancaster PA 17601-5994

Analysis Report**Lancaster Laboratories***Where quality is a science.*

Page: 2 of 4

LLI Sample No. WW 2250404

Collected: 1/18/95 at 08:50 by EA

Submitted: 1/18/95 Reported: 1/31/95

Discard: 3/ 3/95

905 Sheehy Formulation Laboratory Grab Water

Account No: 02423

Argus Research Labs., Inc.

935 Morsham Road

Morsham PA 19044-1230

P.O.
Ref.

CAT NO.	ANALYSIS NAME	AS RECEIVED		
		RESULTS	LIMIT OF QUANTITATION	UNITS
Pesticides/PCB's				
1902	Alpha BHC	< 0.01	0.01	ug/l
1903	Beta BHC	< 0.01	0.01	ug/l
0453	Gamma BHC - Lindane	< 0.01	0.01	ug/l
1904	Delta BHC	< 0.01	0.01	ug/l
0454	Heptachlor	< 0.01	0.01	ug/l
0455	Aldrin	< 0.01	0.01	ug/l
1905	Heptachlor Epoxide	< 0.01	0.01	ug/l
1906	DDE	< 0.01	0.01	ug/l
1907	DDD	< 0.01	0.01	ug/l
0478	DDT	< 0.01	0.01	ug/l
0469	Dieldrin	< 0.01	0.01	ug/l
.77	Endrin	< 0.01	0.01	ug/l
	Chlordane	< 0.3	0.3	ug/l
	Toxaphene	< 4.	4.	ug/l
1910	Endosulfan I	< 0.01	0.01	ug/l
1911	Endosulfan II	< 0.01	0.01	ug/l
1912	Endosulfan Sulfate	< 0.03	0.03	ug/l
0638	Endrin Aldehyde	< 0.1	0.1	ug/l
1913	PCB-1016	< 1.	1.	ug/l
1914	PCB-1221	< 1.	1.	ug/l
1915	PCB-1232	< 1.	1.	ug/l
1916	PCB-1242	< 1.	1.	ug/l
1917	PCB-1248	< 1.	1.	ug/l
1918	PCB-1254	< 1.	1.	ug/l
1919	PCB-1260	< 1.	1.	ug/l

EXACT COPY
JF 4-28-95

Questions? Contact your Client Services Representative
Jeannie Jacobson at (717) 656-2300

Respectfully Submitted
Jennifer E. Hess, B.S.
Group Leader: Pesticides/PCBs



Lancaster Laboratories, Inc.
2425 New Holland Pike
Lancaster, PA 17601-5004

Analysis Report**Lancaster Laboratories***Where quality is a science.*

Page: 3 of 4

LLI Sample No. WW 2250404

Collected: 1/18/95 at 08:50 by EA

Submitted: 1/18/95 Reported: 1/31/95

Discard: 3/ 3/95

Account No: 02423

Argus Research Labs., Inc.

935 Horsham Road

Horsham PA 19044-1230

P.O.

Ref.

905 Sheehy Formulation Laboratory Grab Water

CAT NO.	ANALYSIS NAME	AS RECEIVED		
		RESULTS	LIMIT OF QUANTITATION	UNITS
Method 8150 Herbicides				
1857	2,4-D	< 1.	1.	ug/l
1858	2,4,5-TP	< 0.1	0.1	ug/l
5286	2,4,5-T	< 0.1	0.1	ug/l
5287	Dalapon	< 1.	1.	ug/l
5288	Dinoseb	< 1.	1.	ug/l
5289	Dicamba	< 0.1	0.1	ug/l
5290	MCPP	< 50.	50.	ug/l
5291	MCPA	< 50.	50.	ug/l
5292	2,4-DF (Dichlorprop)	< 1.	1.	ug/l
5293	2,4-DB	< 1.	1.	ug/l

EXACT COPY

JAN 4 1995

Questions? Contact your Client Services Representative
 Jeannie Jacobson at (717) 656-2300

Respectfully Submitted
 Jennifer E. Hess, B.S.
 Group Leader Pesticides/PCBs



Lancaster Laboratories Inc.
 2425 New Holland Pike
 Lancaster PA 17601-5904

Analysis Report**Lancaster Laboratories***Where quality is a science.*

Page: 4 of 4

LLI Sample No. WW 2250404

Collected: 1/18/95 at 08:50 by EA

Submitted: 1/18/95 Reported: 1/31/95

Discard: 3/ 3/95

905 Sheehy Formulation Laboratory Grab Water

Account No: 02423
 Argus Research Labs., Inc.
 935 Morsham Road
 Morsham PA 19044-1230

P.O.
 Rel.

		AS RECEIVED		
CAT			LIMIT OF	
NO.	ANALYSIS NAME	RESULTS	QUANTITATION	UNITS
Anion Scan				
0602	Bromide	< 3.	3.	mg/l
0603	Chloride	< 1.	1.	mg/l
0604	Fluoride	< 0.5	0.5	mg/l
0605	Nitrate Nitrogen	< 0.5	0.5	mg/l
0606	Nitrite Nitrogen	< 0.5	0.5	mg/l
0607	Ortho-phosphate	< 5.	5.	mg/l
0608	Sulfate	< 3.	3.	mg/l

EXACT COPY

Page 4 of 4

Questions? Contact your Client Services Representative
 Jeannie Jacobson at (717) 656-2300

Respectfully Submitted
 Samuel Huber, B.S.
 Group Contr. Inst. Water Qual.



Lancaster Laboratories Inc.
 2425 New Holland Pike
 Lancaster PA 17601-5994

ANALYTICAL LABORATORIES, INC.
P.O. Box 319
CHALFONT, PA 18914
(215) 723-6466

SAMPLE ANALYSIS REPORT

Customer: Argus Research Inc.
~~938 Horsham Rd. 905 Shady Dr~~
Horsham, PA 19440

Sample number : 5393-95D
Date sampled : 05/19/95
Time sampled : 0915
Date received : 05/19/95
Sampled by : Customer
Settlement date:

Attn: 443-8710

Sample source: Room 18

ANALYTICAL RESULTS

Water Safety Classification: No Coliform bacteria were detected. Therefore, the water supply, at the time of sampling, meets the EPA and DER drinking water standards for this parameter.

<u>Parameter</u>	<u>MCL</u>	<u>Result</u>
Coliform Bacteria counts/100 ml	< 1	< 1
Noncoliform Bacteria counts/100 ml		< 1

John F. Brunett
5/31/95

All results which are outside the "Maximum Contaminant Level" (MCL) established under the "Safe Drinking Water Act" are marked by asterisks (**).

Symbol key:
< - less than
TNTC - too numerous to count (> 200)
mg/l - milligram/liter
+ - action level

DER #09-332

EX-100
200-95

Maryann E. Fedock
Maryann E. Fedock/ President

ANALYTICAL LABORATORIES, INC.
P.O. Box 319
CHALFONT, PA 18914
(215) 723-6466

SAMPLE ANALYSIS REPORT

Customer: Argus Research Inc.

~~938 Horsham Rd.~~ 905 Steady Dr
5/31/95 Horsham, PA 19440

Sample number : 5393-95C

Date sampled : 05/19/95

Time sampled : 0915

Date received : 05/19/95

Sampled by : Customer

Settlement date:

Attn: 443-8710

Sample source: Chem. Lab

ANALYTICAL RESULTS

Water Safety Classification: No Coliform bacteria were detected. Therefore, the water supply, at the time of sampling, meets the EPA and DER drinking water standards for this parameter.

<u>Parameter</u>	<u>MCL</u>	<u>Result</u>
Coli Form (D) 05 5/31/95 iform Bacteria. counts/100 ml	< 1	< 1
...coliform Bacteria. counts/100 ml		< 1
Non-coliform (D) 05 5/31/95		

John F. Barnett
5/31/95

All results which are outside the "Maximum Contaminant Level" (MCL) established under the "Safe Drinking Water Act" are marked by asterisks (**).

Symbol key:

< - less than
TNTC - too numerous to count (> 200)
mg/l - milligram/liter
+ - action level

DER #09-332

EXACT COPY
3/6/2095

Maryann E. Fedock
Maryann E. Fedock/ President

ANALYTICAL LABORATORIES, INC.
P.O. Box 319
CHALFONT, PA 18914
(215) 723-6466

SAMPLE ANALYSIS REPORT

Customer: Argus Research Inc.
~~938 Horsham Rd.~~ 905 Shady Dr. *②/JS*
Horsham, PA 19440 *8/2/95*

Sample number: 3561-95B
Date Sampled: 06/22/95
Time Sampled: 1305
Date received: 06/22/95
Sampled by: GWK
Settlement Date:
PO#:

Attn: 443-8710

Sample source: Room 13

ANALYTICAL RESULTS

Water Safety Classification: No Coliform bacteria were detected. Therefore, the water supply, at the time of sampling, meets the EPA and DER drinking water standards for this parameter.

<u>Parameter</u>	<u>MCI</u>	<u>Result</u>
Coliform Bacteria, counts/100 ml	< 1	< 1
Noncoliform Bacteria, counts/100 ml		< 1

*Analytical Laboratories Inc. was again
reminded that they have to
perform Chlorine analysis
also JS 7/31/95*

Symbol key:
< - less than
TNIC - too numerous to count (> 200)
> - greater than

PA DER #09-332

Maryann E. Fedock
Maryann E. Fedock/ President

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MP 8/21/95

ANALYTICAL LABORATORIES, INC.
P.O. Box 319
Chalfont, PA 18914
(215) 723-6460

SAMPLE ANALYSIS REPORT

Customer: Argus Research Inc.

~~938 Horsham Rd.~~ 905 Shady Dr. *Dr. [Signature]*
Horsham, PA 19440 *8/2/95*

Attn: 443-8710

Sample source: Chemistry Lab

Sample Number : 3561-95A
Date Sampled : 06/22/95
Time Sampled : 1305
Date Received : 06/22/95
Sampled By : GWK
Settlement date:

ANALYTICAL RESULTS

Water Safety Classification: No Coliform bacteria were detected. Therefore, the water supply, at the time of sampling, meets the EPA and DER drinking water standards for this parameter.

Parameter	MCL	Result
Total Coliform Bacteria, counts/100 ml	< 1	< 1
Fecal Coliform Bacteria, counts/100 ml	< 1	< 1
Total Chlorine, mg/l as Cl	< 0.1	< 0.1

Analytical Laboratories Inc. was again reminded that they have to perform Chlorine analysis also.
JB 7/31/95

Results which are outside the "Maximum Contaminant Level" (MCL) established under the "Safe Drinking Water Act" are marked with asterisks (**).

Symbol key:

INTC - Too numerous to count (> 200)
< - Less than
mg/l - milligrams/liter
+ - Action Level

EXACT COPY
9/18/95

A DER #09-332

[Signature]
Maryann E. Fedock / President

APPENDIX 6
HISTORICAL CONTROL DATA

SUMMARY OF REPRODUCTIVE INDICES

PERIOD JANUARY 1992 - JANUARY 1994

NUMBER OF STUDIES 105

NUMBER OF RATS:

TESTED	1993
PREGNANT	1802
FOUND DEAD	6
ABORTED	0
DELIVERED	0

NUMBER OF RATS PREGNANT AT
CAESAREAN-SECTIONING 1778

	MEAN or %	RANGE/STUDY MEAN or %
% PREGNANT	91.3	(50.0-100)
AVERAGE # CORPORA LUTEA	18.0	(11.0-21.0)
AVERAGE # IMPLANTATIONS	15.4	(8.3-18.9)
AVERAGE LITTER SIZE		
AVERAGE # LIVE FETUSES	14.6	(8.3-17.2)
AVERAGE # DEAD FETUSES	0.0	--
AVERAGE # RESORPTIONS	0.8	(0-3.3)
AVERAGE # EARLY RESORPTIONS	0.8	(0-3.3)
AVERAGE # LATE RESORPTIONS	0.0	(0-0.2)
AVERAGE % DAMS WITH ANY RESORPTIONS	48.1	(0-83.3)
AVERAGE % DAMS WITH ALL CONCEPTUSES RESORBED	0.1	(0-5.0)

SUMMARY OF REPRODUCTIVE INDICES
CD RAT

	MEAN or %	RANGE/STUDY MEAN or %
AVERAGE % DAMS WITH ONE OR MORE LIVE FETUSES	99.9	(95.0-100)
AVERAGE SEX RATIO, (% MALES/LITTER)	50.1	(38.0-58.5)
AVERAGE FETAL BODY WEIGHT (G)	3.52	(3.20-4.04)
AVERAGE FOR MALES (G)	3.61	(3.21-4.15)
AVERAGE FOR FEMALES (G)	3.42	(3.10-3.92)
AVERAGE % DEAD OR RESORBED CONCEPTUSES/LITTER	5.1	(0-21.9)

SUMMARY OF MATERNAL NECROPSY OBSERVATIONS
CD RAT

PERIOD	JANUARY 1992 - JANUARY 1994
# STUDIES	150
# RATS TESTED	2794
# RATS PREGNANT	2505
# RATS DIED	11
(DEATH ATTRIBUTED TO INTUBATION ACCIDENT)	0
# RATS ABORTED	0
# RATS DELIVERED	683
# RATS WITH 100% RESORPTION	1
# DAMS WITH SINGLE CONCEPTUS LITTER *	
LIVE:	4
RESORBED:	1
ABORTED:	0

EXTERNAL OBSERVATIONS	N	MEAN %	RANGE N	/STUDY %
Red substance around nose and/or mouth	2	0.07	0-1	(0-6.7)
Ear, swollen and purple	2	0.07	0-2	(0-6.9)
Incisors misaligned broken and/or missing	1	0.04	0-1	(0-3.4)
Nose appeared broken	1	0.04	0-1	(0-3.3)
Eye: Red substance in anterior chamber	1	0.04	0-1	(0-4.0)
Calvaria: Red and white raised area	1	0.04	0-1	(0-3.4)
Enophthalmia	1	0.04	0-1	(0-4.0)
Abdominal distension	1	0.04	0-1	(0-6.7)
Head: Mass on left side, contents tan	1	0.04	0-1	(0-4.2)

GROSS LESIONS

GENERAL

Body: Situs inversus, viscera totalis	1	0.04	0-1	(0-4.5)
--	---	------	-----	---------

SKULL

Depressed	1	0.04	0-1	(0-4.0)
-----------	---	------	-----	---------

* Excludes litters that were naturally delivered

SUMMARY OF MATERNAL NECROPSY OBSERVATIONS
CD RAT

GROSS LESIONS	N	MEAN	RANGE	/STUDY
BRAIN				
Cerebrum: Left side, hemorrhagic area	1	0.04	0-1	(0-5.9)
NECK				
Extensive hemorrhaging ventral to cervical vertebrae and around esophagus and trachea	1	0.04	0-1	(0-4.0)
THORAX				
Red mass present	1	0.04	0-1	(0-5.9)
Dark red fluid present	1	0.04	0-1	(0-6.7)
LUNGS				
Discolored	3	0.11	0-2	(0-13.3)
MAMMARY GLAND				
Missing, right inguinal	1	0.04	0-1	(0-5.0)
AXILLA				
Mass present	1	0.04	0-1	(0-4.0)
LIVER				
Enlarged	1	0.04	0-1	(0-5.9)
STOMACH/INTESTINE				
Fundic portion, dark red areas present	1	0.04	0-1	(0-5.0)
Cecum, gas-filled	1	0.04	0-1	(0-4.3)
ABDOMINAL CAVITY				
Umbilical hernia	1	0.04	0-1	(0-4.0)
BACK				
Mass present, gelatinous, tan and/or lobulated	2	0.07	0-2	(0-8.0)

SUMMARY OF MATERNAL NECROPSY OBSERVATIONS
CD RAT

GROSS LESIONS		N	MEAN	RANGE /STUDY	
			%	N	%
KIDNEY					
	Pelvis, slight/ moderate dilation with or without fluid	15	0.54	0-3	(0-16.7)
	Enlarged and tan	1	0.04	0-1	(0-12.5)
	Cyst(s) present	2	0.07	0-1	(0-5.0)
	Raised white areas	1	0.04	0-1	(0-2.9)
SPLEEN					
	Adhesions present between spleen and omentum	1	0.04	0-1	(0-4.3)
	Enlarged	2	0.07	0-1	(0-5.9)
	Enlarged with yellow areas throughout; spleen adhered to pancreas	1	0.04	0-1	(0-4.0)
URETERS					
	Dilated	1	0.04	0-1	(0-4.2)
OVARY					
	Surrounded by a clear fluid-filled cyst	2	0.07	0-1	(0-5.0)
	Parovarian cyst	1	0.04	0-1	(0-6.7)
	Bursa, fluid-filled	1	0.04	0-1	(0-3.3)
VAGINA/CERVIX					
	Cervix distended with fluid	1	0.04	0-1	(0-3.3)
	Vagina contained a brown gelatinous substance	1	0.04	0-1	(0-4.0)
UTERUS					
	Hydrometra	1	0.04	0-1	(0-12.5)
	Distended with fluid	1	0.04	0-1	(0-3.3)
	Hydrometra, yellow debris present, large implantation site	1	0.04	0-1	(0-6.2)
	Contained one fetus	1	0.04	0-1	(0-5.0)
	Right uterine horn adhered to mass	1	0.04	0-1	(0-2.9)

SUMMARY OF MATERNAL NECROPSY OBSERVATIONS
CD RAT

GROSS LESIONS	N	MEAN	RANGE	/STUDY
		%	N	%
INGUINAL AREA				
Mass present	3	0.11	0-2	(0-5.9)
LIMBS				
Left forelimb, dis-				
location between				
carpals and metacarpals	1	0.04	0-1	(0-4.3)
Hindlimb, fracture of				
tibia and fibula	2	0.07	0-1	(0-4.3)
Hindlimb, swollen	1	0.04	0-1	(0-2.9)

SUMMARY OF FETAL EXTERNAL ALTERATIONS
CD RAT

PERIOD JANUARY 1992 - JANUARY 1994

# STUDIES INCLUDED	100
# LITTERS EXAMINED	1710
# LIVE FETUSES EXAMINED	24907

ALTERATION			N	%	RANGE /STUDY	
			N	%		%
HEAD						
	Exencephaly	L	5	0.29	0-1	(0-4.8)
		F	5	0.02	0-1	(0-0.3)
	Meningocele	L	1	0.06	0-1	(0-4.5)
		F	1	0.00	0-1	(0-0.3)
	Cleft lip	L	1	0.06	0-1	(0-4.5)
		F	1	0.00	0-1	(0-0.3)
	Facial cleft	L	1	0.06	0-1	(0-4.3)
		F	1	0.00	0-1	(0-0.3)
EYES						
	Eye bulges	L	12	0.70	0-2	(0-14.3)
	depressed	F	12	0.05	0-2	(0-1.1)
	Eye lid(s) open	L	4	0.23	0-2	(0-9.1)
		F	4	0.02	0-2	(0-0.6)
EARS						
	Low-set	L	5	0.29	0-1	(0-7.1)
		F	5	0.02	0-1	(0-0.6)
PALATE						
	Cleft	L	7	0.41	0-1	(0-4.8)
		F	7	0.03	0-1	(0-0.3)
TONGUE						
	Protrudes	L	3	0.18	0-1	(0-4.8)
		F	3	0.01	0-1	(0-0.3)
JAWS						
	Agnathia	L	3	0.18	0-1	(0-7.1)
		F	3	0.01	0-1	(0-0.6)
	Micrognathia	L	6	0.35	0-1	(0-7.1)
		F	6	0.02	0-1	(0-0.6)

L: LITTER INCIDENCE

F: FETAL INCIDENCE

SUMMARY OF FETAL EXTERNAL ALTERATIONS
CD RAT

ALTERATION				RANGE /STUDY	
		N	%	N	%
BODY					
Edema	L	7	0.41	0-1	(0-7.1)
	F	8	0.03	0-2	(0-0.6)
Umbilical hernia	L	6	0.35	0-1	(0-16.7)
	F	6	0.02	0-1	(0-1.1)
Spina bifida	L	2	0.12	0-1	(0-4.8)
	F	2	0.01	0-1	(0-0.3)
Torso short	L	5	0.29	0-1	(0-4.5)
	F	5	0.02	0-1	(0-0.4)
Gastroschisis	L	1	0.06	0-1	(0-4.5)
	F	1	0.00	0-1	(0-0.4)
Meningocele, thoracic area	L	1	0.06	0-1	(0-4.5)
	F	1	0.00	0-1	(0-0.4)
LIMBS					
Rotated	L	1	0.06	0-1	(0-4.5)
	F	1	0.00	0-1	(0-0.4)
Short	L	1	0.06	0-1	(0-4.5)
	F	1	0.00	0-1	(0-0.3)
Hindlimbs extended towards tail	L	1	0.06	0-1	(0-3.6)
	F	1	0.00	0-1	(0-0.2)
PAWS					
Hind paws, syndactyly	L	1	0.06	0-1	(0-4.5)
	F	1	0.00	0-1	(0-0.3)
Both hind paws, one digit absent	L	1	0.06	0-1	(0-4.5)
	F	1	0.00	0-1	(0-0.3)
GENITAL TUBERCULE					
No skin covering	L	1	0.06	0-1	(0-4.5)
	F	1	0.00	0-1	(0-0.3)
TAIL					
Threadlike	L	7	0.41	0-1	(0-4.8)
	F	7	0.03	0-1	(0-0.4)
Shortened and/or kinked at end	L	2	0.12	0-1	(0-4.5)
	F	2	0.01	0-1	(0-0.3)
Coiled	L	1	0.06	0-1	(0-4.5)
	F	1	0.00	0-1	(0-0.3)
Hematoma	L	1	0.06	0-1	(0-5.0)
	F	1	0.00	0-1	(0-0.3)

L: LITTER INCIDENCE

F: FETAL INCIDENCE

SUMMARY OF FETAL SOFT TISSUE ALTERATIONS
CD RAT

PERIOD JANUARY 1992 - JANUARY 1994
STUDIES INCLUDED 43
LITTERS EXAMINED 948
FETUSES EXAMINED 6664

ALTERATION		RANGE/STUDY			
		N	%	N	%
BRAIN					
Dilated lateral and 3rd ventricles (slight)	L	1	0.11	0-1	(0-5.6)
	F	1	0.02	0-1	(0-0.8)
Dilated 3rd ventricle (moderate)	L	2	0.21	0-1	(0-5.6)
	F	2	0.03	0-1	(0-0.8)
Dilated lateral ventricles (moderate)	L	1	0.11	0-1	(0-5.6)
	F	1	0.02	0-1	(0-0.8)
Dilated lateral ventricles (marked)	L	2	0.21	0-1	(0-5.6)
	F	2	0.03	0-1	(0-0.8)
EYE(S)					
Microphthalmia	L	3	0.32	0-1	(0-7.1)
	F	3	0.05	0-1	(0-1.1)
NASAL PASSAGES					
Moderate dilation	L	1	0.11	0-1	(0-4.8)
	F	1	0.02	0-1	(0-0.6)
HEART					
Situs inversus	L	5	0.53	0-1	(0-5.6)
	F	5	0.08	0-1	(0-0.8)
Ectopic	L	1	0.11	0-1	(0-4.3)
	F	1	0.02	0-1	(0-0.7)
Ventricular septal defect	L	3	0.32	0-1	(0-4.8)
	F	3	0.05	0-1	(0-0.6)
VESSELS					
Situs inversus	L	3	0.32	0-1	(0-5.0)
	F	3	0.05	0-1	(0-0.8)
DIAPHRAGM					
Diaphragmatic hernia	L	2	0.21	0-1	(0-4.3)
	F	2	0.03	0-1	(0-0.7)

L: LITTER INCIDENCE
F: FETAL INCIDENCE

SUMMARY OF FETAL SOFT TISSUE ALTERATIONS
CD RAT

ALTERATION	RANGE/STUDY			
	N	%	N	%
STOMACH				
Situs inversus	L	4	0.42	0-1 (0-5.0)
	F	4	0.06	0-1 (0-0.8)
KIDNEY(S)				
Dilated renal pelvis (slight)	L	5	0.53	0-2 (0-9.1)
	F	6	0.09	0-3 (0-2.0)
Dilated renal pelvis (moderate)	L	15	1.58	0-2 (0-10.0)
	F	16	0.24	0-2 (0-1.3)
Dilated renal pelvis (marked)	L	1	0.11	0-1 (0-3.7)
	F	1	0.02	0-1 (0-0.5)

L: LITTER INCIDENCE

F: FETAL INCIDENCE

SUMMARY OF FETAL SKELETAL ALTERATIONS
CD RAT

PERIOD JANUARY 1992 - JANUARY 1994
STUDIES INCLUDED 43
LITTERS EXAMINED 951
FETUSES EXAMINED 7123

	ALTERATION		N	%	RANGE/STUDY	
SKULL			N	%	N	%
	Nasal(s): short	L	1	0.11	0-1	(0-4.3)
		F	2	0.03	0-2	(0-1.1)
	Frontal(s): incompletely or	L	2	0.21	0-1	(0-4.8)
	not ossified	F	2	0.03	0-1	(0-0.6)
	Parietal(s): incompletely or	L	1	0.11	0-1	(0-4.8)
	not ossified	F	1	0.01	0-1	(0-0.6)
	Interparietal(s):	L	1	0.11	0-1	(0-4.8)
	incompletely or not ossified	F	1	0.01	0-1	(0-0.6)
	Squamosal and Zygomatic:	L	1	0.11	0-1	(0-4.8)
	duplicated	F	1	0.01	0-1	(0-0.6)
	Mandible(s): short and/or	L	5	0.53	0-1	(0-7.1)
	fused	F	6	0.08	0-2	(0-1.1)
	Maxillae: short	L	1	0.11	0-1	(0-4.3)
		F	2	0.03	0-2	(0-1.1)
	Orbit: small	L	3	0.32	0-1	(0-7.1)
		F	3	0.04	0-1	(0-1.1)
	Supraoccipitals: not ossified	L	1	0.11	0-1	(0-4.8)
		F	1	0.01	0-1	(0-0.6)
	Tympanic Ring: not ossified	L	1	0.11	0-1	(0-4.8)
		F	1	0.01	0-1	(0-0.6)
	Palate: incompletely ossified	L	4	0.42	0-1	(0-7.1)
		F	4	0.06	0-1	(0-1.1)
VERTEBRAE						
	Cervical: Arches, open	L	1	0.11	0-1	(0-4.8)
		F	1	0.01	0-1	(0-0.6)
	Thoracic: Centra, bifid	L	54	5.68	0-4	(0-22.2)
		F	60	0.84	0-6	(0-4.6)
	: Centra, unilateral	L	9	0.95	0-1	(0-5.9)
	ossification	F	10	0.14	0-2	(0-1.5)
	: Centra, fused	L	1	0.11	0-1	(0-4.0)
		F	1	0.01	0-1	(0-0.5)

L: LITTER INCIDENCE

F: FETAL INCIDENCE

SUMMARY OF FETAL SKELETAL ALTERATIONS
CD RAT

ALTERATION		N		RANGE/STUDY	
VERTEBRAE (CONT.)					
Thoracic (cont.)					
: Centra, asymmetric	L	1	0.11	0-1	(0-5.6)
	F	1	0.01	0-1	(0-0.8)
: Hemivertebra	L	2	0.21	0-1	(0-5.6)
	F	2	0.03	0-1	(0-0.8)
: Arches, open	L	1	0.11	0-1	(0-4.8)
	F	1	0.01	0-1	(0-0.6)
: Arch, small	L	1	0.11	0-1	(0-4.2)
	F	1	0.01	0-1	(0-0.5)
: Arch, not ossified	L	1	0.11	0-1	(0-4.0)
	F	1	0.01	0-1	(0-0.5)
: Less than 12 present	L	2	0.21	0-1	(0-5.6)
	F	2	0.03	0-1	(0-0.8)
Lumbar:					
Centra, unilateral ossification	L	1	0.11	0-1	(0-4.5)
	F	1	0.01	0-1	(0-0.6)
: Centra, incompletely or not ossified	L	1	0.11	0-1	(0-4.5)
	F	1	0.01	0-1	(0-0.6)
: Centrum, bifid	L	2	0.21	0-1	(0-7.1)
	F	2	0.03	0-1	(0-1.1)
: Hemivertebra	L	1	0.11	0-1	(0-4.3)
	F	1	0.01	0-1	(0-0.6)
: Arches, incompletely or not ossified	L	13	1.37	0-4	(0-17.4)
	F	15	0.21	0-4	(0-2.5)
: Arches, open	L	1	0.11	0-1	(0-4.8)
	F	1	0.01	0-1	(0-0.6)
: None present	L	1	0.11	0-1	(0-4.3)
	F	1	0.01	0-1	(0-0.6)
: Arch, small	L	2	0.21	0-1	(0-4.5)
	F	2	0.03	0-1	(0-0.6)
: Less than 6 present	L	1	0.11	0-1	(0-4.0)
	F	1	0.01	0-1	(0-0.5)
Sacral:					
None present	L	1	0.11	0-1	(0-4.3)
	F	1	0.01	0-1	(0-0.6)
: Less than 3 present	L	2	0.21	0-1	(0-4.2)
	F	2	0.03	0-1	(0-0.5)

L: LITTER INCIDENCE

F: FETAL INCIDENCE

SUMMARY OF FETAL SKELETAL ALTERATIONS
CD RAT

ALTERATION		N		%		RANGE/STUDY	
		N		%			
VERTEBRAE (CONT.)							
Sacral (cont.)							
:	Hemivertebra	L	1	0.11	0-1	(0-3.6)	
		F	1	0.01	0-1	(0-0.5)	
:	Arch, small	L	1	0.11	0-1	(0-3.6)	
		F	1	0.01	0-1	(0-0.5)	
Caudal:	None present	L	3	0.32	0-1	(0-4.3)	
		F	3	0.04	0-1	(0-0.6)	
RIBS							
	Cervical Rib(s) present	L	31	3.26	0-3	(0-13.6)	
		F	34	0.48	0-3	(0-2.1)	
	One or more, wavy	L	43	4.52	0-4	(0-20.0)	
		F	57	0.80	0-10	(0-7.0)	
	One or more, incompletely ossified (hypoplastic), or not ossified	L	22	2.31	0-3	(0-14.3)	
		F	29	0.41	0-4	(0-2.8)	
	Two or more, fused	L	2	0.21	0-1	(0-5.6)	
		F	2	0.03	0-1	(0-0.8)	
	One or more, short	L	1	0.11	0-1	(0-4.8)	
		F	1	0.01	0-1	(0-0.6)	
	Absent	L	1	0.11	0-1	(0-4.2)	
		F	1	0.01	0-1	(0-0.5)	
	Split	L	2	0.21	0-1	(0-4.5)	
		F	2	0.03	0-1	(0-0.6)	
	Proximate	L	1	0.11	0-1	(0-5.6)	
		F	1	0.01	0-1	(0-0.8)	
	Less than 12 present	L	3	0.32	0-1	(0-5.6)	
		F	3	0.04	0-1	(0-0.8)	
MANUBRIUM							
	Duplicated	L	3	0.32	0-1	(0-4.3)	
		F	4	0.06	0-2	(0-1.1)	

L: LITTER INCIDENCE

F: FETAL INCIDENCE

SUMMARY OF FETAL SKELETAL ALTERATIONS
CD RAT

ALTERATION	N	%	RANGE/STUDY	
			N	%
STERNEBRAE				
One or more incompletely ossified or not ossified	L 104	10.94	0-10	(0-43.5)
	F 131	1.84	0-12	(0-7.6)
One or more asymmetric	L 1	0.11	0-1	(0-4.0)
	F 1	0.01	0-1	(0-0.5)
Duplicated	L 3	0.32	0-1	(0-4.3)
	F 4	0.06	0-2	(0-1.1)
Fused	L 1	0.11	0-1	(0-4.3)
	F 1	0.01	0-1	(0-0.6)
XIPHOID				
Duplicated	L 1	0.11	0-1	(0-3.6)
	F 1	0.01	0-1	(0-0.5)
CLAVICULAE				
Wavy	L 1	0.11	0-1	(0-4.5)
	F 1	0.01	0-1	(0-0.6)
SCAPULAE				
Small and irregularly shaped	L 1	0.11	0-1	(0-4.5)
	F 1	0.01	0-1	(0-0.6)
PELVIS				
Pubis(es) and/or Ischium(a): incompletely or not ossified	L 124	13.04	0-7	(0-41.2)
	F 189	2.65	0-21	(0-15.6)
Pubis(es): incompletely ossified	L 108	11.36	0-7	(0-41.2)
	F 167	2.34	0-20	(0-14.8)
Pubis(es): not ossified	L 7	0.74	0-2	(0-8.0)
	F 7	0.10	0-2	(0-1.1)
Ischium(a): incompletely or not ossified	L 53	5.57	0-5	(0-21.7)
	F 64	0.90	0-7	(0-4.4)
Ilium: irregularly shaped	L 1	0.11	0-1	(0-4.5)
	F 1	0.01	0-1	(0-0.6)
LIMBS				
Humerus, Radius and Ulna: short	L 1	0.11	0-1	(0-4.5)
	F 1	0.01	0-1	(0-0.6)
Tibia and Fibula: short	L 1	0.11	0-1	(0-4.3)
	F 2	0.03	0-2	(0-1.1)
Femur, Tibia and Fibula: short	L 1	0.11	0-1	(0-4.5)
	F 1	0.01	0-1	(0-0.6)

L: LITTER INCIDENCE

F: FETAL INCIDENCE

SUMMARY OF FETAL OSSIFICATION SITES
SKELETAL AVERAGES
CD RAT
(CAESAREAN-SECTIONED DAY 20 GESTATION)

PERIOD: JANUARY 1992 - JANUARY 1994

# STUDIES INCLUDED	37
# LITTERS EXAMINED	804
# FETUSES EXAMINED	6045

SKELETAL AVERAGES	FETUS/LITTER	
	MEAN	RANGE/STUDY
HYOID	0.92	(0.84-0.98)
VERTEBRAE:		
CERVICAL	7.00	--
THORACIC	13.04	(13.00-13.29)
LUMBAR	5.95	(5.70-6.00)
SACRAL	3.00	--
CAUDAL	4.99	(4.21-5.28)
RIBS (pairs)	13.03	(13.00-13.22)
STERNUM		
MANUBRIUM	1.00	(0.99-1.00)
STERNAL CENTERS	3.65	(3.35-3.87)
XIPHOID	0.99	(0.96-1.00)
FOREPAWS (Calculated as average per limb)		
CARPALS	0.00	--
METACARPALS	3.53	(3.14-3.72)
DIGITS	5.00	--
PHALANGES	5.09	(4.90-5.40)
HINDPAWS (Calculated as average per limb)		
TARSALS	0.00	--
METATARSALS	3.99	(3.96-4.00)
DIGITS	5.00	--
PHALANGES	4.96	(4.79-5.00)

SUMMARY OF FETAL OSSIFICATION SITES
SKELETAL AVERAGES
CD RAT
(CAESAREAN-SECTIONED DAY 21 GESTATION)

PERIOD: JANUARY 1992 - JANUARY 1994
STUDIES INCLUDED 6
LITTERS EXAMINED 146
FETUSES EXAMINED 1070

SKELETAL AVERAGES	FETUS/LITTER	
	MEAN	RANGE/STUDY
HYOID	1.00	--
VERTEBRAE:		
CERVICAL	7.00	--
THORACIC	13.05	(13.00-13.16)
LUMBAR	5.94	(5.82-6.00)
SACRAL	3.00	--
CAUDAL	7.61	(7.27-7.83)
RIBS (pairs)	13.04	(13.00-13.12)
STERNUM		
MANUBRIUM	1.00	--
STERNAL CENTERS	3.98	(3.95-4.00)
XIPHOID	1.00	--
FOREPAWS (Calculated as average per limb)		
CARPALS	0.00	--
METACARPALS	4.00	(3.99-4.00)
DIGITS	5.00	--
PHALANGES	7.69	(7.44-7.84)
HINDPAWS (Calculated as average per limb)		
TARSALS	0.01	(0.00-0.02)
METATARSALS	4.72	(4.65-4.75)
DIGITS	5.00	--
PHALANGES	5.94	(5.64-6.20)

APPENDIX 7

QUALITY ASSURANCE UNIT FINAL REPORT STATEMENT



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QUALITY ASSURANCE UNIT FINAL REPORT STATEMENT

Study Director: John A. Foss, Ph.D.

Executive Director of Research: Mildred S. Christian, Ph.D., ATS

Protocol 720-002: Developmental Toxicity Study of Hyamine® 1622 in Rats

The draft protocol for this study was audited for adherence to U.S. Environmental Protection Agency (EPA FIFRA) Good Laboratory Practice Standards on 02 Mar 95.

Critical phases of this study were inspected four times; study information and raw data were audited twice (see tables 1 and 2 for dates and phases/data).

The draft final report and the raw data for this study were compared and audited for accuracy, for adherence to protocol requirements, and for adherence to U.S. Environmental Protection Agency (EPA FIFRA) Good Laboratory Practice Standards between 13 Aug and 13 Sept 95, and for inclusion of revisions requested by the Sponsor on 26 Oct 1995.

This study was conducted according to U.S. Environmental Protection Agency (EPA FIFRA) Good Laboratory Practice Standards.

Kathleen A. Moran 26 OCT 95
Kathleen A. Moran, B.A. Date
Manager of Regulatory Compliance

Lisa A. Zaborowski (ben) 26 OCT 95
Lisa A. Zaborowski, B.S. Date
Quality Assurance Associate
and Principal Auditor

QA REPORT TABLE 1
CRITICAL PHASES INSPECTED

Cohabitation

Date of inspection: 23 May 95

Date results reported to the Study Director and Management: 23 May 95

Test Substance Preparation

Date of inspection: 23 May 95

Date results reported to the Study Director and Management: 23 May 95

Test Substance Administration - Gavage

Date of inspection: 05 Jun 95

Date results reported to the Study Director and Management: 06 Jun 95

Caesarean-Sectioning

Date of inspection: 13 Jun 95

Date results reported to the Study Director and Management: 27 Jun 95

QA REPORT TABLE 2

RAW DATA AUDIT(S)

The following study information and raw data were audited from 27 Jun to 06 Jul 95:

- Animal receipt, randomization, physical examination and acclimation.
- Veterinary examination.
- In-life transaction record.
- Feed consumption.
- Cohabitation.
- Caesarean-sectioning.
- Maternal gross observations.
- Fetal gross observations.
- Fetal fixative assignment.
- Fetal visceral examination.
- Fetal skeletal examination.
- Necropsy.
- Organ weights.
- Tissue packing lists.
- Male breeder colony records.
- General comments.
- Study maintenance records.
- Tempscribes.
- Feed and water analyses.
- Edit requests.
- Dosage volumes.

The results of this audit were reported to the Study Director and Management on 06 Jul 95.

The following study information and raw data were audited on 02 Jul 95:

- Vehicle use.
- Test substance receipt, preparation and use.
- Test substance packing lists.

The results of this audit were reported to the Study Director and Management on 06 Jul 95.