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78N-0038

CP 14

Clinical Study Report

CRA: Kathy Wiandt

Date: June 12, 2001

Study Statistician: Jeanne Philipppo

Retention Limit: Until Superseded

Approved by: WZB 6/26/01

Subject: Results of Efficacy Evaluation of Two Handsoap Products and Two Towlette Products in a Modified Healthcare Personnel Handwash Study Versus *Escherichia coli* – CRB-01-05-066-HB / HT# 01-108592-11.

Objective:

The objective of this study was to determine the ability of four antibacterial products to significantly reduce transient microbial flora (*Escherichia coli* 11229) on the hands after a single treatment and after ten (10) treatments. Treatment comparisons were made between the two handwipe products and between the two handwash products.

Materials Tested:

Test Code	Test Material	Active Ingredient	Master Formula Number
A	Handwash Product	0% Salicylic Acid	SWH160-152
B	Handwash Product	2% Salicylic Acid	SWH160-155
C	Handwipe Product	1% Salicylic Acid	SWH94-136
D	Handwipe Product	0% Salicylic Acid	SWH94-137

Key Conclusions:

- All four treatments significantly reduced the level of *E. coli* on the hands after one product application versus baseline.
- All four treatments significantly reduced the level of *E. coli* on the hands after ten product applications versus baseline.
- After one wash, the 2.0% Salicylic acid handwash product had a significantly higher reduction in log counts versus the test placebo (p-value=0.001).
- After ten washes, the 2.0% Salicylic acid handwash had a significantly higher reduction in log counts versus the test placebo (p-value=0.0001).
- After one wash, there was no significant difference between the 1.0% Salicylic acid handwipe and the placebo handwipe product.
- After ten washes, the 1.0% Salicylic acid handwipe had a significantly higher reduction in log counts versus the test placebo (p-value=0.0044).

The summary of the mean logs recovered and the log reductions achieved following the first and tenth washes were determined.

Table I - Summary of HCPHWT Log₁₀ Bacterial Results

Treatment	Sample Size	Baseline	Log ₁₀ Counts - 1 Wash			Log ₁₀ Counts - 10 Washes		
		Mean	Mean	Change from Baseline	% Reduction	Mean	Change from Baseline	% Reduction
A-Handsoap 0% Salicylic Acid	16	6.72	3.91	2.80	99.84	3.80	2.92	99.88
B-Handsoap 2% Salicylic Acid	16	6.81	3.52	3.29	99.95	3.00	3.80	99.98
C-Handwipe 1% Salicylic Acid	16	6.66	4.22	2.44	99.64	3.48	3.18	99.93
D-Handwipe 0% Salicylic Acid	16	6.63	4.34	2.30	99.49	4.19	2.44	99.64

Attached are tables containing the statistical summary of the study results.

Study Summary:

Test Site: Hill Top Research, Miamiville, Ohio

Study Dates: May 22 - June 6, 2001

Investigator: Gayle K. Mulberry, M.S.

Experimental Design: This was a randomized clinical study consisting of a four day test period and a follow-up visit. Four test products were evaluated. Sixteen subjects were used to evaluate each product. Each subject participated in a single test day and a follow-up visit.

Efficacy Measurements Taken: The subjects' hands were contaminated with a suspension of *Escherichia coli* ATCC 11229. Subjects' hands were contaminated eleven times and sampled three times using a plastic bag sampling procedure. The first contamination and sampling was for the determination of the base count. The second contamination and sampling was for determination of the test count after one treatment with the assigned test product. After eleven contamination steps and ten treatments with the assigned test product the hands were sampled using the plastic bag sampling procedure.

Subject Demographics: Sixty-four (64) male and female subjects, ≥ 18 years old, who do not regularly use antibacterial/antimicrobial soaps, medicated lotions or creams and or antidandruff shampoos were enrolled into the study. Sixteen subjects were used to evaluate one of four test products.

Overview: To become familiar with the wash procedure using a liquid hand soap, the subjects assigned to the handwash products practiced the wash procedure with Baby-san®. To become familiar with the wipe procedure, subjects assigned to the handwipe products practiced the wipe procedure with Nice 'n' Clean®. For the base count, subjects' hands were contaminated with *E. coli*. Immediately following the contamination step, the organisms on the subjects' hands were removed using a plastic bag sampling procedure.

Prior to each treatment wash, subjects' hands were contaminated with *E. coli*. After completing the contamination step, the subjects performed the test product application procedure with the assigned test product. For the subjects assigned to the hand wash products, the subjects lathered their hands for fifteen seconds and rinsed their hands for thirty seconds. For the subjects assigned to the handwipe product, the subjects wiped each hand for fifteen seconds. Approximately five minutes following the product treatment procedure, the organisms on both of the subjects' hands were removed using a plastic bag sampling procedure. Approximately five minutes following the tenth treatment, the organisms on the subjects' hands were removed using a plastic bag sampling procedure.

Samples of the subjects' sampling solutions were diluted, plated, and incubated. Following incubation, the numbers of colony forming units (CFU's) were enumerated. Antibacterial activity was determined by comparing the number of bacteria removed from the hands after one treatment with the assigned test product and ten treatments with the assigned Test Product to the number of bacteria removed from unwashed hands.

Data Analysis:

The investigator was responsible for statistical analysis. For the bag juice results, each subject's base sampling CFU's was compared to their test sampling CFU's using a nonparametric Wilcoxon paired signed-rank test. P-values ≤ 0.05 were considered statistically significant. Percent change for each organism was computed by the following formula:

$$1 - \left(\frac{\text{geometric mean of the test CFU's}}{\text{geometric mean of the baseline CFU's}} \right) \times 100$$

Treatment comparisons were analyzed by a Wilcoxon-Mann-Whitney Test using Exact methods.

Regulatory/Ethics Status:

This study was conducted in compliance with federal, state, and local regulations, guidelines, and standards including those related to Informed Consent and Good Clinical Practices as specified under 21 CFR 321.66. This study was conducted with IRB approval.

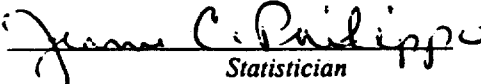
Subject Accountability:

Eighty subjects were screened for the study. Sixty-four subjects were screened, enrolled and completed this study. Sixteen subjects were excluded from the study.

Adverse Events:

There was one adverse event in this study. Subject #35 reported a head cold on 6/5/01. The adverse event was not related to the product treatment. The adverse event was resolved on 6/19/01.


Clinical Research Associate


Statistician

HTR Study Number 01-108592-11
Table 1A. CFU count summary statistics by HTR Code.

10:55 Wednesday, June 6, 2001

HTR Code	Sample Size	Base Count			Test Count 1			Test Count 2		
		Mean	Median	Std. Error	Mean	Median	Std. Error	Mean	Median	Std. Error
HTR Code A	16	6.07E+06	6.25E+06	7.62E+05	1.26E+04	1.15E+04	2.63E+03	7.60E+03	5.92E+03	1.26E+03
HTR Code B	16	6.93E+06	7.35E+06	5.91E+05	4.19E+03	3.62E+03	5.49E+02	1.68E+03	1.38E+03	3.69E+02
HTR Code C	16	5.82E+06	6.46E+06	7.68E+05	3.76E+04	3.53E+04	9.42E+03	9.54E+03	4.84E+03	3.51E+03
HTR Code D	16	4.78E+06	4.31E+06	5.03E+05	3.73E+04	3.27E+04	6.87E+03	2.57E+04	1.76E+04	5.93E+03

Table 1B. Log10(Count) summary statistics by HTR Code.

HTR Code	Sample Size	Base Count			Test Count 1			Test Count 2		
		Mean	Median	Std. Error	Mean	Median	Std. Error	Mean	Median	Std. Error
HTR Code A	16	6.7168	6.7957	0.0656	3.9120	4.0203	0.1128	3.7950	3.7580	0.0627
HTR Code B	16	6.8064	6.8648	0.0441	3.5155	3.5559	0.0950	3.0042	3.1257	0.1168
HTR Code C	16	6.6587	6.8059	0.0963	4.2173	4.5297	0.1944	3.4826	3.6610	0.2040
HTR Code D	16	6.6329	6.6197	0.0481	4.3366	4.4844	0.1433	4.1915	4.2367	0.1230

Table 1C. Log10 changes from baseline summary statistics and percent reductions.

HTR	Code	Sample Size	Test Count 1				Test Count 2			
			Mean Change	Median Change	Std. Error Change	Percent Reduction	Mean Change	Median Change	Std. Error Change	Percent Reduction
HTR	Code A	16	-0.0000	-0.0000	0.0000	99.84	-0.0000	-0.0000	0.0000	99.88
HTR	Code B	16	-0.0000	-0.0000	0.0000	99.90	-0.0000	-0.0000	0.0000	99.98
HTR	Code C	16	-0.0000	-0.0000	0.0000	99.94	-0.0000	-0.0000	0.0000	99.93
HTR	Code D	16	-0.0000	-0.0000	0.0000	99.94	-0.0000	-0.0000	0.0000	99.61

HTR Study Number 01-108592-11
Table 2A. Listing of CFU/ml counts by HTR Code.

10:55 Wednesday, June 6, 2001

HTR Code	Subject	Base Count			Test Count 1			Test Count 2		
		Left CFU/ml	Right CFU/ml	Avg. CFU/ml	Left CFU/ml	Right CFU/ml	Avg. CFU/ml	Left CFU/ml	Right CFU/ml	Avg. CFU/ml
HTR Code A	1	6.25E+06	6.00E+06	6.13E+06	1.96E+04	1.44E+04	1.70E+04	7.57E+03	7.52E+03	7.55E+03
	7	4.15E+06	4.40E+06	4.28E+06	5.26E+03	3.76E+03	4.51E+03	7.27E+03	4.30E+03	5.79E+03
	11	8.65E+06	9.50E+06	9.08E+06	2.14E+04	1.86E+04	2.00E+04	1.86E+04	1.15E+04	1.51E+04
	14	2.35E+06	2.18E+06	2.27E+06	2.09E+03	5.03E+03	3.56E+03	2.80E+03	6.69E+03	4.75E+03
	17	8.15E+06	5.55E+06	6.85E+06	1.93E+04	7.08E+03	1.32E+04	1.14E+04	9.80E+03	1.06E+04
	24	2.95E+06	3.85E+06	3.40E+06	8.50E+02	2.32E+03	1.59E+03	5.38E+03	3.19E+03	4.29E+03
	27	2.52E+06	2.25E+06	2.39E+06	1.15E+03	1.46E+03	1.31E+03	5.37E+03	3.03E+03	4.20E+03
	32	7.20E+06	9.30E+06	8.25E+06	9.00E+03	9.80E+03	9.40E+03	5.45E+03	2.93E+03	4.19E+03
	34	6.40E+06	6.35E+06	6.38E+06	6.46E+03	6.88E+03	6.67E+03	5.71E+03	4.38E+03	5.05E+03
	38	1.38E+07	1.16E+07	1.27E+07	4.60E+04	3.95E+04	4.28E+04	1.96E+04	2.48E+04	2.22E+04
	44	9.90E+06	9.20E+06	9.55E+06	2.45E+04	1.23E+04	1.84E+04	7.44E+03	1.40E+04	1.07E+04
	48	4.50E+06	6.15E+06	5.33E+06	5.49E+03	1.42E+04	9.85E+03	4.56E+03	7.55E+03	6.06E+03
	50	6.65E+06	9.35E+06	8.00E+06	2.07E+04	2.05E+04	2.06E+04	6.77E+03	8.85E+03	7.81E+03
	53	5.35E+06	3.30E+06	4.33E+06	1.56E+04	1.34E+04	1.45E+04	2.26E+03	2.96E+03	2.61E+03
	59	6.55E+06	7.25E+06	6.90E+06	1.58E+04	1.61E+04	1.60E+04	1.55E+03	5.84E+03	3.70E+03
	62	1.24E+06	1.45E+06	1.35E+06	1.61E+03	2.37E+03	1.99E+03	6.40E+03	7.65E+03	7.03E+03
		6.04E+06	6.11E+06	6.07E+06	1.34E+04	1.17E+04	1.26E+04	7.38E+03	7.81E+03	7.60E+03

Table 2A. Listing of CFU/ml counts by HTR Code.

HTR Code	Subject	Base Count			Test Count 1			Test Count 2		
		Left CFU/ml	Right CFU/ml	Avg. CFU/ml	Left CFU/ml	Right CFU/ml	Avg. CFU/ml	Left CFU/ml	Right CFU/ml	Avg. CFU/ml
HTR Code B	1	4.40E+06	4.10E+06	4.25E+06	6.38E+03	6.09E+03	6.23E+03	9.40E+02	1.87E+03	1.41E+03
	7	4.30E+06	1.11E+07	1.04E+07	9.40E+03	8.15E+03	8.78E+03	3.97E+03	3.75E+03	3.86E+03
	11	4.50E+06	4.40E+06	4.45E+06	5.54E+03	4.14E+03	4.84E+03	1.46E+03	1.24E+03	1.35E+03
	14	4.40E+06	4.40E+06	4.40E+06	5.31E+03	1.43E+03	1.57E+03	1.28E+03	1.60E+02	7.20E+02
	17	4.40E+06	4.40E+06	4.40E+06	1.99E+03	3.36E+03	2.68E+03	2.61E+03	2.06E+03	2.34E+03
	24	4.90E+06	7.05E+06	6.08E+06	7.20E+03	5.70E+03	6.45E+03	3.57E+03	5.94E+03	4.76E+03
	26	4.55E+06	5.10E+06	4.83E+06	2.45E+03	1.39E+03	1.92E+03	5.93E+03	3.02E+03	4.48E+03
	29	8.50E+06	9.60E+06	9.05E+06	1.80E+03	4.65E+03	3.23E+03	1.04E+03	4.10E+02	7.25E+02
	35	9.35E+06	9.20E+06	9.28E+06	2.12E+03	2.83E+03	2.48E+03	4.30E+02	1.80E+02	3.05E+02
	40	6.80E+06	8.35E+06	7.58E+06	5.07E+03	1.81E+03	3.44E+03	9.50E+02	2.21E+03	1.58E+03
	43	7.50E+06	9.80E+06	8.65E+06	3.89E+03	2.44E+03	3.17E+03	6.50E+02	2.30E+02	4.40E+02
	47	2.26E+06	3.30E+06	2.78E+06	4.72E+03	4.69E+03	4.71E+03	1.28E+03	4.20E+02	8.50E+02
	51	6.85E+06	7.40E+06	7.13E+06	3.11E+03	4.04E+03	3.58E+03	8.00E+01	1.60E+02	1.20E+02
	54	5.85E+06	5.35E+06	5.60E+06	4.06E+03	3.28E+03	3.67E+03	1.77E+03	1.92E+03	1.85E+03
	57	1.06E+07	1.06E+07	1.06E+07	5.50E+03	9.75E+03	7.63E+03	1.24E+03	2.24E+03	1.74E+03
	64	3.65E+06	7.95E+06	5.80E+06	1.10E+02	3.20E+02	2.15E+02	1.80E+02	5.70E+02	3.75E+02
		6.34E+06	7.32E+06	6.93E+06	4.17E+03	4.21E+03	4.19E+03	1.71E+03	1.65E+03	1.68E+03

Table 2A. Listing of CFU/ml counts by HTR Code.

HTR Code	Subject	Base Count			Test Count 1			Test Count 2		
		Left CFU/ml	Right CFU/ml	Avg. CFU/ml	Left CFU/ml	Right CFU/ml	Avg. CFU/ml	Left CFU/ml	Right CFU/ml	Avg. CFU/ml
HTR Code C	2	3.04E+06	2.71E+06	2.88E+06	6.60E+03	1.24E+04	9.50E+03	3.69E+03	7.34E+03	5.52E+03
	5	6.10E+06	8.15E+06	7.13E+06	5.15E+04	6.70E+04	5.93E+04	2.12E+04	1.36E+04	1.74E+04
	9	8.70E+06	7.45E+06	8.08E+06	3.00E+04	7.95E+04	5.48E+04	3.03E+03	5.15E+03	4.09E+03
	13	2.58E+06	2.44E+06	2.51E+06	2.42E+03	3.00E+02	1.36E+03	1.96E+03	5.40E+02	1.25E+03
	18	3.60E+06	3.80E+06	3.70E+06	3.70E+04	2.42E+04	3.06E+04	8.20E+03	6.55E+03	7.38E+03
	23	2.70E+05	3.30E+05	3.00E+05	9.30E+02	2.00E+01	4.75E+02	4.00E+01	1.00E+02	7.00E+01
	25	7.60E+06	8.15E+06	7.88E+06	6.50E+04	2.70E+04	4.60E+04	5.95E+04	4.95E+04	5.45E+04
	31	5.70E+06	5.20E+06	5.45E+06	2.27E+04	1.33E+04	1.80E+04	7.70E+02	4.30E+02	6.00E+02
	33	2.37E+06	2.02E+06	2.20E+06	3.40E+04	5.15E+04	4.28E+04	4.80E+03	6.74E+03	5.77E+03
	39	5.85E+06	8.05E+06	6.95E+06	5.25E+04	2.70E+04	3.98E+04	5.80E+02	5.70E+02	5.75E+02
	41	5.25E+06	9.90E+06	7.58E+06	1.16E+04	5.66E+03	8.63E+03	3.11E+03	5.23E+03	4.17E+03
	46	8.55E+06	9.75E+06	9.15E+06	4.25E+04	7.70E+04	5.98E+04	1.21E+04	1.30E+04	1.26E+04
	52	3.00E+06	3.00E+06	3.00E+06	3.54E+03	3.61E+03	3.58E+03	1.70E+02	9.00E+01	1.30E+02
	55	6.40E+06	5.55E+06	5.98E+06	1.22E+05	1.90E+05	1.56E+05	2.67E+04	2.76E+04	2.72E+04
	58	8.75E+06	9.60E+06	9.18E+06	4.30E+04	3.24E+04	3.77E+04	3.50E+03	1.20E+03	2.35E+03
	61	1.24E+07	9.85E+06	1.11E+07	4.45E+04	2.12E+04	3.29E+04	1.10E+04	7.22E+03	9.11E+03
HTR Code C		5.64E+06	6.00E+06	5.82E+06	3.56E+04	3.95E+04	3.76E+04	1.00E+04	9.05E+03	9.54E+03

Table 2A. Listing of CFU/ml counts by HTR Code.

HTR Code	Subject	Base Count			Test Count 1			Test Count 2		
		Left CFU/ml	Right CFU/ml	Avg. CFU/ml	Left CFU/ml	Right CFU/ml	Avg. CFU/ml	Left CFU/ml	Right CFU/ml	Avg. CFU/ml
HTR Code D	3	1.04E+06	2.00E+06	2.57E+06	1.02E+05	5.25E+04	7.73E+04	2.83E+03	2.38E+03	2.61E+03
	5	1.04E+06	1.05E+06	8.05E+06	5.95E+04	4.05E+04	7.50E+04	9.70E+04	5.05E+04	7.38E+04
	10	1.04E+06	1.04E+06	1.74E+06	3.70E+04	2.78E+04	3.24E+04	3.15E+04	3.70E+04	3.43E+04
	11	1.04E+06	1.04E+06	1.04E+06	1.05E+04	1.05E+04	2.00E+04	1.05E+04	2.75E+04	2.51E+04
	12	1.04E+06	1.04E+06	1.04E+06	1.05E+04	1.05E+04	3.31E+04	1.05E+04	2.00E+04	2.13E+04
	21	5.30E+06	6.50E+06	5.90E+06	5.15E+04	4.25E+04	4.70E+04	2.17E+04	1.82E+04	2.00E+04
	28	3.40E+06	3.05E+06	3.23E+06	1.46E+04	6.08E+03	1.03E+04	1.10E+04	1.00E+04	1.05E+04
	30	5.90E+06	6.10E+06	6.00E+06	4.25E+03	1.51E+04	9.68E+03	1.28E+04	1.75E+04	1.52E+04
	36	3.85E+06	3.65E+06	3.75E+06	2.53E+04	2.50E+04	2.52E+04	1.00E+04	9.05E+03	9.53E+03
	37	5.05E+06	7.15E+06	6.10E+06	4.30E+04	7.30E+04	5.80E+04	5.05E+04	6.70E+04	5.88E+04
	42	2.75E+06	3.38E+06	3.07E+06	6.59E+03	2.59E+03	4.59E+03	1.25E+03	1.44E+03	1.35E+03
	45	6.40E+06	8.20E+06	7.30E+06	3.90E+04	6.75E+04	5.33E+04	1.74E+04	1.20E+04	1.47E+04
	49	2.44E+06	3.50E+06	2.97E+06	1.16E+04	1.16E+04	1.16E+04	4.51E+03	7.29E+03	5.90E+03
	56	5.55E+06	3.80E+06	4.68E+06	1.20E+05	6.00E+04	9.00E+04	4.85E+04	2.35E+04	3.60E+04
	60	6.45E+06	6.75E+06	6.60E+06	5.35E+04	2.04E+04	3.70E+04	7.35E+03	6.34E+03	6.85E+03
	63	1.61E+06	2.21E+06	1.91E+06	1.20E+02	2.34E+03	1.23E+03	9.20E+04	5.35E+04	7.28E+04
HTR Code D		4.24E+06	5.32E+06	4.78E+06	4.01E+04	3.44E+04	3.73E+04	2.86E+04	2.28E+04	2.57E+04

Table 2B. Listing of Log10 counts and results of the Wilcoxon Paired Sign Rank Test.

HTR Code	Subject	-----Test Count 1-----			Log Diff. (Base-Test)	Wilcoxon p-value
		Left Log10 CFU/ml	Right Log10 CFU/ml	Avg. Log10 CFU/ml		
HTR Code A	1	4.2923	4.1584	4.2253	2.5617	
	7	3.7210	3.5752	3.6481	2.9827	
	11	4.3304	4.2695	4.3000	2.6574	
	14	3.3201	3.7016	3.5109	2.8439	
	17	4.2856	3.8500	4.0678	2.7599	
	24	2.9294	3.3655	3.1475	3.3802	
	27	3.0607	3.1644	3.1125	3.2643	
	32	3.9542	3.9912	3.9727	2.9402	
	34	3.8102	3.8376	3.8239	2.9806	
	38	4.6628	4.5966	4.6297	2.4725	
	44	4.3892	4.0899	4.2395	2.7402	
	48	3.7396	4.1523	3.9459	2.7751	
	50	4.3160	4.3118	4.3139	2.5830	
	53	4.1931	4.1271	4.1601	2.4633	
	59	4.1987	4.2068	4.2027	2.6355	
	62	3.2068	3.3747	3.2908	2.8366	0.0001*
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HTR Code A		3.9006	3.9233	3.9120	2.8048	0.0001*
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Table 2B. Listing of Log10 counts and results of the Wilcoxon Paired Sign Rank Test.

HTR Code	Subject	-----Test Count 2-----			Log Diff. (Base-Test)	Wilcoxon p-value
		Left Log10 CFU/ml	Right Log10 CFU/ml	Avg. Log10 CFU/ml		
HTR Code A	1	3.8791	3.8762	3.8777	2.9094	
	7	3.8615	3.6335	3.7475	2.8832	
	11	4.2695	4.0607	4.1651	2.7923	
	14	3.4472	3.8254	3.6363	2.7185	
	17	4.0569	3.9912	4.0241	2.8037	
	24	3.7308	3.5038	3.6173	2.9104	
	27	3.7300	3.4814	3.6057	2.7711	
	32	3.7364	3.4669	3.6016	3.3113	
	34	3.7566	3.6415	3.6991	3.1054	
	38	4.2923	4.3945	4.3434	2.7588	
	44	3.8716	4.1461	4.0089	2.9709	
	48	3.6590	3.8779	3.7685	2.9526	
	50	3.8306	3.9469	3.8888	3.0081	
	53	3.3541	3.4713	3.4127	3.2107	
	59	3.1903	3.7664	3.4784	3.3599	
	62	3.8062	3.8837	3.8449	2.2825	0.0001*
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HTR Code A		3.7795	3.8105	3.7950	2.9218	0.0001*
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Positive difference indicates reduction from baseline.
* Indicates significance

Table 2B. Listing of Log10 counts and results of the Wilcoxon Paired Sign Rank Test.

HTR Code	Subject	-----Test Count 1-----			Log Diff. (Base-Test)	Wilcoxon p-value
		Left Log10 CFU/ml	Right Log10 CFU/ml	Avg. Log10 CFU/ml		
HTR Code B	4	3.8048	3.7839	3.7944	2.8782	
	6	3.9731	3.9112	3.9421	3.0706	
	10	3.5490	3.6474	3.5982	3.2812	
	16	3.7251	3.6464	3.6857	2.7840	
	19	3.2989	3.5263	3.4126	3.4705	
	22	3.8573	3.7559	3.8066	2.9687	
	26	3.3892	3.1430	3.2661	3.4167	
	29	3.2553	3.6675	3.4614	3.4945	
	35	3.3263	3.4518	3.3891	3.5782	
	40	3.7050	3.2577	3.4813	3.3958	
	43	3.5899	3.3874	3.4887	3.4445	
	47	3.6739	3.6712	3.6726	2.7638	
	51	3.4928	3.6064	3.5496	3.3029	
	54	3.6085	3.5159	3.5622	3.1856	
	57	3.7404	3.9890	3.8647	3.1606	
	64	2.0414	2.5051	2.2733	4.4581	0.0001*
HTR Code B		3.5019	3.5291	3.5155	3.2909	0.0001*

HTR Code	Subject	-----Test Count 2-----			Log Diff. (Base-Test)	Wilcoxon p-value
		Left Log10 CFU/ml	Right Log10 CFU/ml	Avg. Log10 CFU/ml		
HTR Code B	4	2.9731	3.2718	3.1225	3.5501	
	6	3.5988	3.5740	3.5864	3.4263	
	10	3.1644	3.0934	3.1289	3.7505	
	16	3.1072	2.2041	2.6557	3.8141	
	19	3.4166	3.3139	3.3653	3.5179	
	22	3.5527	3.7738	3.6632	3.1120	
	26	3.7731	3.4800	3.6265	3.0563	
	29	3.0170	2.6128	2.8149	4.1409	
	35	2.6335	2.2553	2.4444	4.5229	
	40	2.9777	3.3444	3.1611	3.7160	
	43	2.8129	2.3617	2.5873	4.3458	
	47	3.1072	2.6232	2.8652	3.5711	
	51	1.9031	2.2041	2.0536	4.7989	
	54	3.2480	3.2833	3.2656	3.4821	
	57	3.0934	3.3502	3.2218	3.8035	
	64	2.2553	2.7559	2.5056	4.2258	0.0001*
HTR Code B		3.0396	2.9689	3.0042	3.8021	0.0001*

Positive difference indicates reduction from baseline.

* Indicates significance

10:55 Wednesday, June 6, 2001

Table 2B. Listing of Log10 counts and results of the Wilcoxon Paired Sign Rank Test.

		-----Test Count 1-----			Log Diff. (Base-Test)	Wilcoxon p-value
HTR Code	Subject	Left Log10 CFU/ml	Right Log10 CFU/ml	Avg. Log10 CFU/ml		
HTR Code C	2	3.8195	4.0934	3.9565	2.5014	
	5	4.7118	4.8261	4.7689	2.0793	
	9	4.4771	4.9004	4.6887	2.2171	
	13	3.3838	2.4771	2.9305	3.4690	
	18	4.5682	4.3838	4.4760	2.0920	
	23	2.9685	1.3010	2.1348	3.3402	
	25	4.8129	4.4314	4.6221	2.2738	
	31	4.3560	4.1239	4.2399	2.4960	
	33	4.5315	4.7118	4.6216	1.7184	
	39	4.7202	4.4314	4.5758	2.2607	
	41	4.0645	3.7528	3.9086	2.9493	
	46	4.6284	4.8865	4.7574	2.2030	
	52	3.5490	3.5575	3.5533	2.9239	
	55	5.0864	5.2788	5.1826	1.5927	
	58	4.6335	4.5105	4.5720	2.3901	
	61	4.6484	4.3263	4.4873	2.5561	0.0001*
HTR Code C		4.3100	4.1245	4.2173	2.4414	0.0001*
		-----Test Count 2-----			Log Diff. (Base-Test)	Wilcoxon p-value
HTR Code	Subject	Left Log10 CFU/ml	Right Log10 CFU/ml	Avg. Log10 CFU/ml		
HTR Code C	5	4.3263	4.1335	4.2299	2.0183	
	9	3.4814	3.7118	3.5966	3.3092	
	13	3.2923	2.7324	3.0123	3.3872	
	18	3.9138	3.8162	3.8650	2.7030	
	23	1.6021	2.0000	1.8010	3.6739	
	25	4.7745	4.6946	4.7346	2.1614	
	31	2.8865	2.6335	2.7600	3.9760	
	33	3.6812	3.8287	3.7550	2.5851	
	39	2.7634	2.7559	2.7597	4.0768	
	41	3.4928	3.7185	3.6056	3.2523	
	46	4.0828	4.1139	4.0984	2.8621	
	52	2.2304	1.9542	2.0923	4.3848	
	55	4.4265	4.4409	4.4337	2.3415	
	58	3.5441	3.0792	3.3116	3.6505	
	61	4.0414	3.8585	3.9500	3.0935	0.0001*
HTR Code C		3.5067	3.4586	3.4826	3.1761	0.0001*

Positive difference indicates reduction from baseline.

* Indicates significance

Table 2B. Listing of Log10 counts and results of the Wilcoxon Paired Sign Rank Test.

HTR Code	Subject	-----Test Count 1-----			Log Diff. (Base-Test)	Wilcoxon p-value
		Left Log10 CFU/ml	Right Log10 CFU/ml	Avg. Log10 CFU/ml		
HTR Code D	3	5.0086	4.7202	4.8644	1.5410	
	8	4.7745	4.9566	4.8656	2.0345	
	12	4.5682	4.4440	4.5061	1.9224	
	15	4.3181	4.6075	4.4628	2.3777	
	20	4.7202	4.1335	4.4268	2.1505	
	21	4.7118	4.6284	4.6701	2.0985	
	28	4.1644	3.7839	3.9741	2.5338	
	30	3.6284	4.1790	3.9037	2.8744	
	36	4.4031	4.3979	4.4005	2.1733	
	37	4.6335	4.8633	4.7484	2.0304	
	42	3.8189	3.4133	3.6161	2.8680	
	45	4.5911	4.8293	4.7102	2.1498	
	49	4.0645	4.0645	4.0645	2.4013	
	56	5.0792	4.7782	4.9287	1.7334	
	60	4.7284	4.3096	4.5190	2.3004	
	63	2.0792	3.3692	2.7242	3.5514	0.0001*
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HTR Code D		4.3307	4.3424	4.3366	2.2963	0.0001*
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HTR Code	Subject	-----Test Count 2-----			Log Diff. (Base-Test)	Wilcoxon p-value
		Left Log10 CFU/ml	Right Log10 CFU/ml	Avg. Log10 CFU/ml		
HTR Code D	3	5.0086	3.3700	4.1893	2.6601	
	8	4.7745	4.9566	4.8656	2.0345	
	12	4.5682	4.4440	4.5061	1.9224	
	15	4.3181	4.6075	4.4628	2.3777	
	20	4.7202	4.1335	4.4268	2.1505	
	21	4.7118	4.6284	4.6701	2.0985	
	28	4.0414	4.0000	4.0207	2.4872	
	30	4.1072	4.2430	4.1751	2.6030	
	36	4.0000	3.9566	3.9783	2.5956	
	37	4.7033	4.8261	4.7647	2.0141	
	42	3.0969	3.1584	3.1276	3.3565	
	45	4.2405	4.0792	4.1599	2.7001	
	49	3.6542	3.8627	3.7585	2.7073	
	56	4.6857	4.3711	4.5284	2.1336	
	60	3.8663	3.8021	3.8342	2.9852	
	63	4.9638	4.7284	4.8461	1.4295	0.0001*
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HTR Code D		4.2129	4.1701	4.1915	2.4414	0.0001*
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Positive difference indicates reduction from baseline.
* Indicates significance

Table 3. Results of the Wilcoxon-Mann-Whitney Test for the between treatment analysis of test articles.

Group	-Test Count 1-- p-value	-Test Count 2-- p-value
A vs B	0.0010*	0.0001*
C vs D	0.3080	0.0044*

* indicates significant difference between treatments

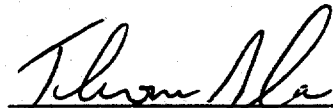
HTR Study No.: 01-108592-11

Sponsor Study No.: CRB-01-05-066-HB

QUALITY ASSURANCE STATEMENT

This study was inspected in accordance with the Standard Operating Procedures of Hill Top Research, Inc. To assure compliance with the study protocol, the Quality Assurance Unit performed an inspection during the conduct of this study and completed an audit of the study records.

Data reviewed by:



Thomas Asplan, A.A.S., B.S.
Auditor, Quality Assurance

6/26/01

Date

CLINICAL STUDY PROTOCOL

Clinical Research & Biometrics Department
Sharon Woods Technical Center
Cincinnati, Ohio 45241

Title: Efficacy Evaluation Of Two Liquid Soap Products and Two Towelette Products
In A Modified Health Care Personnel Handwash Study Versus *Escherichia Coli*

Study Number: CRB-01-05-066-HB / HT# 01-108592-11

Issue Date: 5/15/01

Products Tested: Antibacterial Handsoap Prototype (2% Salicylic Acid)
Control Handsoap Prototype (0% Salicylic Acid)
Antibacterial Handwipe Prototype (1% Salicylic Acid)
Control Handwipe Prototype (0% Salicylic Acid)

Test Facility: Hill Top Research, Inc.
Main and Mill Streets
Miamiaville, Ohio 45147

Principal Investigator: Gayle Mulberry, M.S.

Sub-Investigators: Kathleen A. Baxter, B.S.
Ann R. Brady, A.S.

Test Sponsor: The Procter & Gamble Co., Inc.
Sharon Woods Technical Center
11520 Reed Hartman Highway
Cincinnati, Ohio 45241

Sponsor Toxicologist: Candace Doepker, Ph.D. (513) 626-5536 work
Tim Long, Ph.D. (513) 626-4027 home

Sponsor Representative/CRA: Kathy Wiandt, B.A. (513) 626-5225 (513) 398-6035

Sponsor Statistician: Jeanne Philipppo, B.A. (513) 626-5937

Expected Study Start Date: May 22, 2001

Expected Study End Date: June 6, 2001

I. Study Objective and Background

A. Objective

The objective of the study is to determine the ability of four antibacterial products to significantly reduce transient microbial flora (*Escherichia coli* ATCC 11229) on the hands after a single treatment and after ten (10) treatments. Treatment comparisons will be made between the two handwipe products and between the two handwash treatments.

B. Background

The skin microflora can be divided into two (2) groups, the resident flora and the transient flora. The resident flora includes organisms that are consistently present on the skin. The transient flora are the contaminating skin organisms resulting from contact with the environment. They comprise a wide variety of Gram positive and Gram negative species that can be responsible for the spread of infections and gastrointestinal diseases.

Since the benefits that result from washing with antibacterial soaps can not be easily measured under consumer use conditions, it is necessary to do controlled clinical studies to demonstrate their efficacy. This clinical study is a modification of an ASTM test method, "Evaluation of Health Care Personnel Handwash Formulation"⁽¹⁾ and reported in the Tentative Final Monograph for Health Care Antiseptic Drug Products⁽²⁾. It is used to determine the ability of an antimicrobial handwashing agent, when used in a hand washing procedure, to reduce the transient microbial flora (contaminants). This study is designed to demonstrate the efficacy of four antibacterial products in reducing the numbers of a marker organism, *Escherichia coli* ATCC 11229 on the hands after a contamination and a single handwash and after ten handwashes. Efficacy is determined by comparing the numbers of marker organisms on the hands before and after using the test products.

C. Study Safety Statement

This testing meets the ethical requirements stipulated in the Sponsor's Policy for Research Involving Human Subjects. Appropriate safety testing has been completed and risk assessments justify the placement of the test products in this study at these concentrations (levels of exposure).

II. Study Summary

A. Overview

This randomized clinical study will consist of a four day test period and a follow-up visit. Four (4) test products will be evaluated. Sixty-four (64) male and female subjects, ≥ 18 years old, who do not regularly use antibacterial/antimicrobial soaps, medicated lotions and creams, and or antidandruff shampoos (Appendix E), will be enrolled into the study. Sixteen (16) subjects will be used to evaluate each test product.

On the day of the study, the subjects will report to the clinical test facility. During this period, subjects' hands will be contaminated with a suspension of *E. coli*. Subjects' hands will be contaminated eleven (11) times and sampled three (3) times using a plastic bag sampling procedure. The first contamination and sampling will be for the determination of the base count. The second contamination and sampling will be for determination of the test count after one (1) treatment with the assigned Test Product. After eleven (11) contamination steps and ten (10) treatments with the assigned Test Products the hands will be sampled using the plastic bag sampling procedure

To become familiar with the wash procedure using the liquid hand soap, subjects assigned to handwash products will begin the test procedure by first performing a practice wash with Baby-san®. To become familiar with the wipe procedure using the towelette products, subjects assigned to handwipe products will begin the test procedure by first performing a practice wipe with Nice 'n' Clean®. For the base count, subjects will have their hands contaminated with *E. coli*. Immediately

following the contamination step, the organisms on the subjects' hands will be removed using a plastic bag sampling procedure.

Prior to each treatment wash, subjects' hands will be contaminated with *E. coli*. After completing the contamination step, the subjects will perform the test product application procedure with the assigned Test Product. Approximately five (5) minutes following the first procedure, the organisms on both of the subjects' hands will be removed using a plastic bag sampling procedure. Approximately five (5) minutes following the tenth treatment, the organisms on the subjects' hands will be removed using a plastic bag sampling procedure.

Aliquots of the subjects' sampling solutions will be diluted, plated, and incubated. Following incubation, the number of colony forming units (CFU's) will be enumerated. Antibacterial activity is determined by comparing the number of bacteria removed from the hands after one (1) treatment with the assigned Test Product and ten (10) treatments with the assigned Test Product to the number of bacteria removed from unwashed hands.

B. Study Schedule

1. Subject Qualification and Enrollment

Prospective subjects will visit the test facility to be screened for their eligibility to participate in the study. Eligibility will be based upon information provided in the Demographics/Dermatological/Medical History Form (DCF 1) and the Inclusion/Exclusion Form (DCF 2); and completion of a written informed consent (Appendix A).

2. Test Period

Subjects continuing on the study will be assigned a permanent subject number. Subjects will be assigned to one of the four test products according to the study randomization.

The following outlines the schedule of procedures for the test day:

1. Subjects will perform a practice wash with Baby-san® Handsoap or Nice "n' Clean® Handwipe (Appendix D).
2. Subjects will rinse their hands with 70% alcohol and rinse their hands under running tap water (Section G).
3. Subjects' hands will be contaminated (Section E).
4. Subjects' hands will be sampled for a base count (Section F).
5. Subjects will rinse their hands with water for 30 seconds (Section G).
6. Subjects will rinse their hands with 70% alcohol and rinse with tap water (Section G).
7. Subjects' hands will be contaminated (Section E).
8. Subjects will wash their hands following the wash procedure for the assigned Test Product (Section C, Appendix C).
9. Subjects' right and left hands will be sampled for a treatment value four (4) minutes and thirty (30) seconds \pm thirty (30) seconds after the first wash with the assigned Test Product (Section F).
10. The hands will be rinsed for thirty seconds.
11. Subjects will perform steps 7 and 8 (above) a total of nine (9) more times at a minimum of five (5) minutes between each wash procedure.
12. The subjects' hands will be sampled for a treatment value four (4) minutes and thirty (30) seconds \pm thirty (30) seconds after the tenth wash with the assigned Test Product (Section F).
13. Subjects' hands will be disinfected with a bland soap and water wash and Hibiclens® (4%

chlorhexidine gluconate) wash and with a 70% alcohol rinse (Section G).

Note: *A detailed schedule of the above procedures can be found in Appendix D.*

To ensure that any delayed adverse events, such as primary skin infections, are reported to the Study Investigator, all test subjects will be given a copy of Subjects' Instructions Following Study Completion (Appendix B) before leaving the clinical site after they have completed the study. This sheet will instruct the subjects to examine their hands and wrists daily until the final scheduled visit for the presence of pimples, blisters, or raised, red itching bumps surrounded by erythema and/or edema that may be indicative of a skin infection. Subjects, who notice such lesions, will be instructed to call the clinical test site. The subjects will return to the clinical test site within four (4) to nine (9) days after the study procedures have been completed to have their hands and wrists examined by a technician. The technician will complete DCF 3 for each subject on their follow-up visit.

C. Product Treatment Procedure

Subjects will wash their hands and wrists according to the procedure described in Product Treatment Procedure, Appendix C. In general the following should be noted:

- The temperature should be checked and recorded before each wash.
- The water pressure at each sprayer to be used for the study should flow at 4 L/min.
- Subjects should remove all jewelry from hands and wrists prior to start of wash procedure.
- Water temperature should be maintained at 95 - 100° F.

D. Preparation of Bacterial Suspensions

A stock culture of *Escherichia coli*, ATCC 11229, will be prepared by transferring three (3) isolated colonies from an agar plate or slant aseptically to a tube containing sterile Trypticase Soy Broth (TSB). The inoculated broth will then be incubated for 24 ± 4 hours at $35 \pm 2^\circ$ C. At least three (3) additional 24 hour broth transfers will be made in tubes containing appropriate volumes TSB from this broth culture.

A 2-liter flask containing 1000 mL of TSB will be inoculated with 1.0 mL of the final 24 hour broth transfer. The flask will be incubated for 24 ± 4 hours at $35 \pm 2^\circ$ C. Prior to any withdrawal of culture, whether for hand contamination or for numbers assay, the suspension will be stirred or shaken. The suspension will be assayed for number of organisms at the beginning and end of the treatment period. A suspension will not be used for more than eight (8) hours.

E. Contamination

Note: *Prior to contamination, subjects hands must be visibly dry. Also, care should be taken to ensure that the culture is evenly spread over both hands*

A total volume of 4.5 mL of the assigned bacterial suspension will be dispensed into the subjects' cupped hands in 1.5 mL increments. After each 1.5 mL aliquot is added, the suspension will be rubbed thoroughly over the surface of both hands, not going above the wrist and avoiding the nail beds. Each application and spreading should last approximately twenty (20) seconds. Between each aliquot the hands will be held away from the body and allowed to air dry for approximately thirty (30) seconds. Following the third 1.5 mL aliquot, the hands are allowed to air dry for approximately one (1) minute. A record of base and test contaminations will be documented on Source Document 1 or 2.

F. Bacterial Sampling Procedure

For removal of bacteria from the subjects' hands, loose fitting plastic bags with low bioburden will be placed on each subject's right and left hands. A 75 mL aliquot of stripping solution [0.1% Triton X-100 in 0.075 M phosphate buffer, 1.0% polysorbate (Tween) 80, 0.3% Lecithin, pH 7.9] will be aseptically added into each bag. The same solution will be used for the base counts and test counts.

The bag on each hand will be secured at the wrist with a child's size tourniquet and massaged for one (1) minute in a uniform manner by a lab technician. Aliquots of the solution will be aseptically obtained directly from the bag without touching the hands in the process and will be appropriately diluted in a sterile diluent with the appropriate neutralizer within in one (1) minute of sampling. A record of base and test samplings will be documented on Source Document 1.

The solution samples for bacteria counts will be labeled by either an Investigator derived code or the actual subject's number so that the individuals who prepare the plates and count the CFU's are unaware of the sources of the sampling solution.

G. Disinfection of Hands

After the baseline sampling, the subjects will rinse their hands for thirty (30) seconds under running tap water. The subjects' hands will be disinfected with a 70% alcohol wash. Subjects' hands will be squirted with 70% alcohol for approximately ten (10) seconds. Subjects will rub the alcohol over the surface of their hands and wrists for approximately fifteen (15) seconds. Subjects will rinse their hands and wrists under running tap water for approximately fifteen (15) seconds and dry their hands and wrists with paper towels.

After the final sampling is completed, the subject's hands will be washed with a bland soap (provided by the investigator) for approximately for thirty (30) seconds and rinsed for approximately fifteen (15) seconds. The subjects' hands will then be washed with Hibiclens® (4% chlorhexidine gluconate) for at least sixty (60) seconds. Subjects' hands and wrists will be rinsed with a 70% alcohol wash for ten (10) seconds. The subjects will rub the alcohol on all surfaces of their hands for fifteen (15) seconds and allow their hands to air dry.

A record of each disinfection procedure will be recorded on Source Document 1.

H. Plating and Incubation of the Organisms

The *Escherichia coli* organisms in the sampling solution are to be counted using a standard surface inoculation technique.

Aliquots of dilutions of the base sampling solution from each sample bag representing dilutions of 10^{-4} , 10^{-5} , and 10^{-6} will be plated in duplicate.

Following the wash with the test product, a 1.0 mL aliquot of the previously diluted sampling solution from each sample bag will be plated onto three MacConkey's agar plates (approximately 0.33 mL per plate) to achieve a 10^{-1} dilution. Also, aliquots of dilutions of the sampling solution from each sample bag representing dilutions of 10^{-2} , 10^{-3} , 10^{-4} and 10^{-5} will be plated in duplicate. The MacConkey's agar plates will be incubated 18-24 hours at $35 \pm 2^{\circ}\text{C}$. Standard plate counting procedures will be used to count the CFU's of *E. coli*. In general, the number of CFU's per sample will be determined by taking the average of the counts from the plates which are in the range of ≥ 25 to ≤ 250 CFU's. If there are no plates with counts within this range, the following rules will be used to determine which counts will be used for obtaining the number of CFU's for that specimen:

1. If all of the counts are below the prescribed range, the numbers below 25 from the undiluted plates will be used.

2. If the counts from the highest dilution are > 250 , the numbers, obtained from using the estimated counting procedure described in Appendix F, will be used.

Results will be reported on DCF 4.

III. Study Population

Subjects will be screened for their eligibility to participate based upon information provided in the Demographics/Dermatological/Medical History Form [Data collection form (DCF) 1]. Only subjects meeting the inclusion/exclusion criteria, outlined in DCF 2, will be allowed to participate in the study. If a subject is admitted to this study in apparent violation of any of the above criteria, the reason(s) for admission will be noted by the Investigator or her designee.

A. Subject Inclusion Criteria

Subjects will be eligible for enrollment if they:

1. Are a male or female, over 18 years of age;
2. Have signed a written informed consent (Appendix A);
3. Are in good health, as evidenced by response to the Demographics/Dermatological/ Medical History Form (DCF 1);
4. Have hands and wrists that are free of dermatoses, cuts, lesions, and other skin disorders;
5. Are willing to comply with all study protocol requirements.

B. Subject Exclusion Criteria

Subjects will not be enrolled in the study if they:

1. Are currently participating in another clinical study at this or any other facility;
2. Have participated in any type of arm or hand wash study within the past 7 days;
3. Have cuts, lesions, or other skin disorders on their hands or wrists;
4. Have soap, detergent, antibiotic, and/or perfume allergies;
5. Have eczema or psoriasis on their hands or arms;
6. Are using antibacterial/antimicrobial soaps (liquids and/or bars), medicated lotions and creams, and/or anti-dandruff shampoos in the home within the last week (Appendix E);
7. Have excessively long or artificial nails (≥ 2 mm free edge) which would interfere with sampling;
8. Are currently pregnant;
9. Are currently lactating;
10. Have been diagnosed as having a medical condition which would preclude participation such as: diabetes, hepatitis, an organ transplant, or AIDS (or HIV positive); and/or
11. Have any other medical condition, which in the opinion of the Investigator, would preclude participation.

D. Subject Number Assignment and Randomization

Upon entry into the study, each subject will be assigned a screening number beginning with 1001. Subjects will be assigned a permanent consecutive number, beginning with 001, as they are accepted into the study. This number will be used to identify the subject for the duration of the study.

IV. Study Material

A. Test Product

The test products will be sent by the Sponsor to the clinical site prior to study initiation. The test products will be identified with the appropriate label affixed to the outside of each container.

B. Shipping of Treatment Products and Other Study Supplies

The quantity of all treatment products and other study supplies, shipped to and returned from the clinical site, will be documented by the test site. The treatment products will be packed into one or more cartons labeled with:

1. the study number;
2. distributor statement (i.e., "Distributed by Hill Top Research, Inc." with the facility's full address and phone number);
3. any applicable safety and handling procedures.

C. Return of Study Materials

Upon completion of the study, the Investigator(s) will insure that all test products and study materials, whether completely used, partially used, or unused will be returned to the Sponsor at the following address:

The Procter & Gamble Company
Sharon Woods Technical Center
11520 Reed Hartman Highway
Cincinnati, Ohio 45241
Attn.: Kathy Wiandt

V. Other Study Documentation

A. Adverse Events

Should any unexpected or serious adverse event occur during the clinical study or as a result of test product or study procedures, the subject will be requested to return to the site to be examined by the investigator or designee. The Investigator will determine if the adverse event is likely to be associated with product treatment or the study procedures. The investigator or other qualified medical personnel will determine if the event warrants termination of participation and/or to prescribe treatment, if necessary. The Investigator will notify the Sponsor representatives, Ward L. Billhimer, 513-626-1926 (work) or 513-831-8163 (home) or Kathy Wiandt, 513-626-5225 (work) or 513-398-6035 (home).

Each subject will need to be followed until the resolution of any adverse event. Information pertaining to the presenting signs, working diagnosis, assessment of the relationship of the adverse event to the product treatment, results of the follow-up visits and any prescribed treatment, will be documented in DCF 5. If treatment by a physician is necessary, this treatment will be documented on DCF 6.

The following criteria will be used to determine the reporting time frame.

1. Any serious adverse events or adverse events requiring immediate medical attention will be reported to the Sponsor's Monitor immediately (night or day) by telephone.
2. Adverse events resulting in subject termination from the study will be reported during the immediate business day by telephone.
3. Adverse events that do not require discontinuation of test participation can be reported during the immediate business day or next business day by telephone.

4. In the event of a serious adverse reaction, not necessarily related to use of the test product, or in the event of a death from any cause, the Investigator must report the event to the Sponsor's Monitor and to the IRB as soon as possible..

B. Protocol Amendments

If it becomes necessary to modify this protocol, the modification will be documented by a protocol amendment signed by the investigator, a representative of the Sponsor and approved by the Institutional Review Board.. All amendments to the final protocol will be consecutively numbered and will describe any changes made and the rationale for making the changes.

C. Protocol Deviations

If a deviation from the final protocol occurs, it is the responsibility of the Investigator, or designee, to notify the Clinical Research Associate or designee. The Institutional Review Board will be notified within twenty-four hours of any deviation that poses additional risks to the subjects. The deviation and subsequent notification will be documented appropriately.

D. Study Monitoring

The Investigator will permit a representative of the Sponsor (usually the Clinical Research Associate) to visit the facility during the course of the study to monitor study progress. During the visit(s), the Investigator will permit the monitor to inspect all forms and corresponding study subject's records to verify adherence to the protocol. The study monitor will also be permitted to review and verify test articles, wash procedure, and any Investigator-generated or Sponsor-generated study documents. The monitor will document and discuss this visit with the Investigator, or his designee, including any problems that are to be resolved.

VI. Statistical Analyses

The investigator will be responsible for all statistical analyses. For the bag juice results, each subject's base sampling CFU's will be compared to their test sampling CFU's using a nonparametric Wilcoxon paired signed-rank test. P-values ≤ 0.05 will be considered statistically significant. Percent change for the test organism will be computed, if needed, by the following formula:

$$1 - \frac{(\text{geometric mean of the test CFU's})}{\text{geometric mean of the baseline CFU's}} \times 100$$

Treatment comparisons will be made between the two wash treatments and the two wipe treatments. Treatment comparisons will be analyzed by a Wilcoxon-Mann-Whitney Test using Exact methods.

VII. Investigator Responsibilities

A. Institutional Review Board (IRB) Review and Approval

Review by an IRB is required to conduct this study. A copy of the approval letter along with a list of the IRB members who acted on this protocol and a statement that the IRB is in compliance with current Good Clinical Practices (GCP) regulations will be provided to the Sponsor.

B. Subject Informed Consent

All subjects will be informed as to the type of study, the general nature of the products being tested, and any known or anticipated adverse reactions, which might result from participation. Each subject must provide the Investigator with written informed consent to serve as a participant in the study. Basic elements of informed consent are outlined in 21 CFR 50.25.

C. Final Report

The Sponsor will generate a final report of clinical results. The investigator will provide a detailed description of the adverse events and deviations from the protocol. The investigator will also include an accounting of the subjects screened, eliminated, enrolled and terminated. The Investigator will submit the legible copies of all data collection forms. The Sponsor may request one (1) copy of all data collection forms before the Investigator's report is ready for submission to the Sponsor.

D. Record Retention

The Investigator will retain all study records in accordance with the test facility's SOP's.

E. Confidentiality

The Investigator and employees of the test facility are obligated to keep any information confidential regarding any of the personal cleansing products and all aspects of the study, as subject to the terms and conditions of the Laboratory Services Agreement between the test facility and Sponsor.

VIII. References

1. *Annual Book of ASTM Standards*, Volume 11.04, ASTM Designation: E 1174-94, Standard Test Method for "Evaluation of Health Care Personnel Handwash Formulation".
2. Tentative Final Monograph for Health-Care Antiseptic Drug Products; Proposed Rule, 21 CFR Parts 333 and 369, *Federal Register*, Volume 59, No. 116, June 17, 1994.

IX. Attachments

The following Appendices, Data collection forms are included as attachments to the Final Protocol:

- A Written Informed Consent
- B Subject's Follow-up Instructions
- C Product Treatment
- D Schedule of Test Period Procedures
- E List of Representative Antibacterial/Antimicrobial Products
- F Microbiological Media and Methods

Data Collection Forms

- 1 Demographics/Dermatological/Medical History Form
- 2 Inclusion/Exclusion Form
- 3 Follow-up Visit
- 4 Microbiology Results
- 5 Adverse Event
- 6 Physician's Report Form

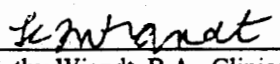
Source Documents

- 1 Treatment Phase (Baseline, Wash 1 and Wash 10)
- 2 Treatment Phase (Washes 2 through 9)

X. Sponsor and Investigator Concurrence

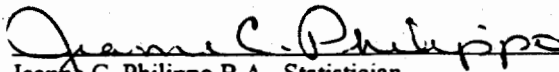
For The Procter and Gamble Company

PREPARED BY:


Kathy Wiandt, B.A., Clinical Research Associate
Clinical Research and Biometrics Department

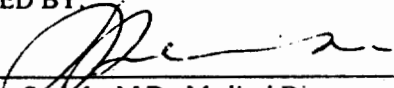
Date: 5/15/01

STATISTICIAN:


Jeanne C. Philippo, B.A., Statistician
Clinical Research and Biometrics Department

Date: 5/15/01

APPROVED BY:

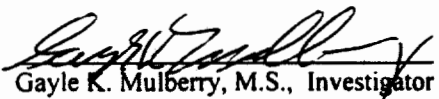

Bruce Semple, M.D., Medical Director
Clinical Research and Biometrics Department

Date: 5/15/01

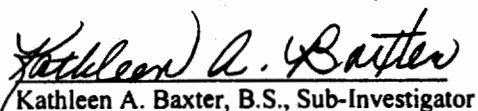
Agreed and Accepted by Hill Top Research, Inc. and the Study Investigator(s) for
CRB-01-05-066-HB:

I certify that I have reviewed and approved the protocol, informed consent form, and other associated documents and agree to abide by their terms. In addition, I agree to conduct this clinical study in compliance with federal, state and local government regulations, guidelines and standards applicable to such studies including, but not limited to, those relating to Institutional Review Board (IRB), Informed Consent, and Good Clinical Practices.

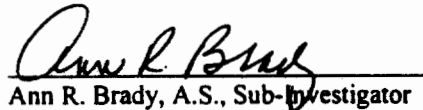
I am aware that it is the responsibility of the Investigator to promptly report to the IRB all changes to the research activity and all unanticipated problems involving risk to human subjects. In addition, as Investigator, I am aware that a summary report must be submitted to the IRB when the study is completed. These guidelines are in accordance with CFR 312.66. The Sponsor will be copied on all correspondence to and from the IRB.


Gayle K. Mulberry, M.S., Investigator

Date: 5-15-01


Kathleen A. Baxter, B.S., Sub-Investigator

Date: 5-15-01


Ann R. Brady, A.S., Sub-Investigator

Date: 5-15-01

Appendix A
HT-01-108592-11
CRB-01-05-066-HB

WRITTEN INFORMED CONSENT

To be provided by the clinical site.

Appendix B
HT-01-108592-11
CRB-01-05-066-HB

SUBJECT'S INSTRUCTIONS FOLLOWING STUDY COMPLETION

You have just completed participation in a clinical study, "Efficacy Evaluation Of Two Liquid Soap Products and Two Towelette Products In A Modified Health Care Personnel Handwash Study Versus *Escherichia coli*". During this study, a quantity of bacteria (*E. coli*) was placed on the surface of both your hands. Although we do not expect you to have any adverse experience as a result of participation in this study, there is a remote possibility that an infection may develop on your hands and wrists within four (4) to nine (9) days.

To determine whether you have developed an infection from the test bacteria, we would like you to examine your hands and wrists daily. If you notice the appearance of any pimples, blisters or raised bumps surrounded by redness and/or swelling, please contact Gayle Mulberry or Ann Brady at (513) 831-3114 during normal business hours (8:00 am-5 p.m.) or at (513) 831-3354 after hours.

You are required to return to the test site for a follow-up visit. Your follow-up is scheduled for:

Date

Time

Thank you for your cooperation.

Appendix C

HT-01-108592-11

CRB-01-05-066-HB

PRODUCT TREATMENT PROCEDURE

Part I: *For subjects assigned to the Liquid Soap Product*

- Water temperature should be maintained at 95 - 100° F.
- The temperature should be checked and recorded before each wash.
- The water pressure at each spigot to be used for the study should flow at 4 L/min.
- Subjects should remove all jewelry from hands and wrists prior to start of wash procedure.

The following wash procedure will be performed by each subject:

1. Subjects will be instructed to wet their hands under the running water.
2. **2.0 mL of product** will be dispensed from a disposable syringe into the subjects' hands by a laboratory technician.
3. The technician will instruct the Subjects to lather all surfaces of their hands and wrists for **fifteen (15) seconds**.
4. Subjects will rinse their hands under running tap water for thirty (30) seconds.
5. A. For test washes #1 and #10, hands will not be dried.
B. For test washes #2 through #9, subjects will dry their hands with paper towels.
(Note: following the practice wash, subjects' hands will be disinfected and contaminated.)
6. Bags will be placed on the subjects' right and left hands for sampling after the first wash and after the tenth treatment. Sampling time will be approximately five (5) minutes following the wash with the test product.

OR

Part II: *For subjects assigned to the Towelette*

1. The technician will dispense the appropriate towelette test product into the subject's left hand using a gloved hand.
2. The subject will rub all surfaces of their right hand and wrist for fifteen (15) seconds while the technician instructs the subject to:
 - rub palm
 - rub back of hand
 - rub fingers and web areas between fingers
 - rub the tips of the fingers
3. The subject will transfer the wipe to their right hand.
4. The subject will rub their left hand and wrist for fifteen (15) seconds while the technician instructs the subjects to:
 - rub palm
 - rub back of hand
 - rub fingers and web areas between fingers
 - rub the tips of the fingers

Appendix D
HT-01-108592-11
CRB-01-05-066-HB

SCHEDULE OF TEST PERIOD PROCEDURES

1. Practice treatment with Test Product:

For subjects assigned to the Liquid Soap Products

- subjects wet hands under running tap water
- dispense 2.0 mL of Baby San® into subjects' hands
- subjects lather hands and wrists for fifteen (15) seconds
- subjects rinse hands under running tap water for thirty (30) seconds
- subjects dry hands with a paper towel

For subjects assigned to the Towelette Products

- towelette is placed in subjects' left hand
- subject will rub all surfaces of their right hands and wrist for 15 seconds including palmar surface, back of hand, fingers and web area between fingers, and finger tips
- subject transfers towelette to right hand
- subject will rub all surfaces of their left hands and wrist for 15 seconds including palmar surface, back of hand, fingers and web area between fingers, and finger tips

2. 70% alcohol rinse

- squirt backs and palms of subjects' hands with 70% alcohol for 10 seconds
- subjects rub alcohol over hands for 15 seconds
- subjects rinse hands under running tap water for 15 seconds
- subjects dry hands with paper towels

3. Base contamination

- dispense 1.5 mL aliquot of bacterial suspension onto both subjects' hands
- subjects rub aliquot over hands for 20 seconds
- allow subjects' hands to air dry for approximately 30 seconds
- repeat application 2 times
- allow subjects' hands to air dry 1 minute after the last application

4. Base sampling

- place bags on subject's right and left hands
- dispense 75 mL stripping solution into each bag
- secure bags
- massage for 1 minute
- sample each bag

5. Water rinse

- subjects rinse hands with water for 30 seconds

6. 70% alcohol rinse

- perform as above

7. Test contamination (prior to Test Product treatments 1 through 10)

- perform as above under base contamination

Appendix D (continued)

HT-01-108592-11

CRB-01-05-066-HB

8. Test Products Treatments (treatments 1 through 10)

- perform as described under practice treatment
- for treatments #1 and #10, hands will not be dried prior to sampling
- for treatments # 2 through #9 subjects will dry hands with paper towels

9. Test sampling - Following Treatment 1

- perform as above under base sampling
- subjects rinse hands with water for 30 seconds after the first test sampling

10. Test sampling - Following Treatment 10

- place bag on of the subject's hands
- dispense 75 mL stripping solution into the bag
- secure bag
- massage for 1 minute
- sample bag

11. Disinfection

- subject rinse hands for thirty (30) seconds
- squirt subjects' hands with 2 mL of bland soap
- subjects wash hands and wrists for approximately 30 seconds
- subjects rinse hands and wrists for approximately 15 seconds
- squirt subjects' hands with 5 mL of Hibiclens®
- subjects wash hands and wrists for at least 60 seconds
- subjects rinse hands and wrists for 15 seconds
- squirt backs, palms and wrists of subjects' hands with 70% alcohol for 10 seconds
- subjects rub alcohol over hands and wrists for 15 seconds
- subjects' hands will be allowed to air dry

Appendix E
HT-01-108592-11
CRB-01-05-066-HB

LIST OF ANTIBACTERIAL / ANTIMICROBIAL PRODUCTS

Medicated Acne Cleansers

Benzac W Wash 5
Desuam-X 5 Wash
Benzac W Wash 10
Desquam-X 10m Wash
Fostex 10% BPO Wash
Oxy 10 Wash
Propa P.H. Liquid Acne Soap
PanOxyl 5
Fostex 10% BPO
PanOxyl 10
Clearasil Antibacterial Soap
Sastid Plain Therapeutic Shampoo and Acne Wash
Oxy Clean Soap
Fostex Medicated Cleansing Bar
Salicylic Acid and Sulfur Soap
Sulfur Soap

Antidandruff Shampoos

Head and Shoulders (all formulas)
Selsun Blue (all formulas)
Pert Plus for Dandruff
Suave for dandruff
Neutrogena T-gel
Neutrogena T-sal
Scalpacin
Tegrin
Any antidandruff shampoo

Anti-bacterial Soaps

Safeguard bar and liquid
Lever 2000 bar and liquid
Irish Spring bar
Dial bar and liquid
Softsoap Antibacterial Soap

Antibiotic Ointments and Creams

Bacitracin
Polysporin
J & J First Aid Cream
Neomycin

Antibacterial Dishwashing Liquids

Dawn
Joy
Dial
Palmolive

Appendix F
HT-01-108592-11
CRB-01-05-066-HB

MICROBIOLOGICAL MEDIA AND METHODS

0.075M Phosphate Buffer Solution with Neutralizers

Weigh 0.4 grams of KH_2PO_4 , 10.1 grams of Na_2HPO_4 , 10.0 grams of Polysorbate (Tween) 80, 3 grams of lecithin, and 1.0 gram of Triton X-100. Dissolve in 1 liter of distilled or deionized water. Adjust to pH 7.9 \pm 0.1 with 1 N HCl or 1 N NaOH. Dispense buffer in bottles so that after autoclaving the volume equals 75 \pm 1 mL. Loosely cap bottles and sterilize in the autoclave at 121°C.

0.0375M Phosphate Buffer Solution with Neutralizers

Weigh 0.2 grams of KH_2PO_4 , 5.05 grams of Na_2HPO_4 , 10.0 grams of Polysorbate (Tween) 80 and 3 grams of lecithin. Dissolve in 1 liter of distilled or deionized water. Adjust to pH 7.9 \pm 0.1 with 1 N HCl or 1 N NaOH. Dispense buffer in appropriate volumes. Loosely cap vessels and sterilize in the autoclave at 121°C.

MacConkey's Agar

Suspend 50 grams in 1 liter of distilled or deionized water. Loosely cap flask and sterilize in the autoclave at 121°C. Cool to 45-50°C in a water bath. Pour in sterile 15 x 100 mm Petri dishes. Allow to cool and solidify on a level flat surface. Check for sterility. Prepared plates are stored at 2 - 8°C and used within 30 days.

Estimated Plate Count Procedure

Do not record counts on crowded plates from the highest dilution as too numerous to count (TNTC). If the number of colonies per plate exceeds 250, count colonies in those portions of the plate that are representative of colony distribution and calculate the Estimated Standard Plate Count (ESPC) from these counts. The ESPC will be determined utilizing the grid embossed area on the lighted surface of the colony counter. Each large square on the grid is 1 cm². If there are fewer than 10 colonies per square centimeter, count colonies in 12 squares, selecting, if representative, six consecutive squares horizontally across the plate and six consecutive squares at right angles, being careful not to count a square more than once. When there are more than 10 colonies per square centimeter, count colonies in four such representative portions. In both instances, multiply the average found per square centimeter by the area of the plate used to determine the estimated number of colonies per plate.

If the total number of CFU's have been estimated according to the procedure described above, ESPC (Estimated Standard Plate Count) should be recorded following the value.

Note: If the highest dilution plated contains >250 CFU's and a count \leq 300 CFU's has been previously determined, that value may be reported. It will not be necessary to estimate the total CFU's on a plate containing >250 CFU's using the above procedure. Plates containing the highest dilution of test specimen plated and the CFU counts are greater than 300, then the above procedure should be used to determine the total CFU count.

Data Collection Form 1
DEMOGRAPHICS/DERMATOLOGICAL/MEDICAL HISTORY FORM

Study #	Hill Top Research, Inc.	Visit Code	Date	Subject Initials	Subject Screen #
01-108592-11 CRB-01-05-066-HB		Subject Qualification	____/____/____ mm dd yy	____/____/____ F M L	Permanent #:

Gender: <input type="checkbox"/> Male <input type="checkbox"/> Female	Age: _____ Years
--	-------------------------

Does the subject have any of the following at the treatment sites?

I. DERMATOLOGIC DISORDER	No	Yes	Don't Know
1. Psoriasis ?			
2. Eczema ?			
3. Skin Cancer ?			
4. Skin Allergies ? Please specify:			
5. Hives ?			

Does the Subject have any of the following (present and past)?

II. OTHER MEDICAL INFORMATION	No	Yes	Don't Know
1. Allergies.? Please specify.			
2. Hepatitis ?			
3. Heart and Vascular Disease?			
4. Liver Disease ?			
5. Kidney Disease ?			
6. Tuberculosis ?			
7. Diabetes ? Controlled? Diet [] Oral [] Insulin []			
8. Cancer ?			
9. Auto-immune disease (Lupus erythematosus, thyroiditis, AIDS, etc.) ?			
10. Organ transplant ?			
11. Any other condition not listed ? Please specify:			

Is the subject taking any medication? If yes, please specify below:

III. MEDICATION	No	Yes	Don't Know
1. Antibiotics, oral or systemic ?			
2. Cortisone, Steroids, ACTH, Anti-reaction Drugs ?			
3. Heart Medication ?			
4. Insulin ?			
5. Other ?			

Comments:

Based on the above medical history, the subject is: ☐ **Qualified** or ☐ **Not qualified** for the study.

Interviewer's Signature:	Date: ____/____/____ mm dd yy
---------------------------------	---

**Data Collection Form 2
INCLUSION / EXCLUSION FORM**

Study #	Hill Top Research, Inc.	Visit Code	Date	Subject Initials	Subject Screen #
01-108592-11 CRB-01-05-066-HB		Subject Qualification	mm dd / yy	F / M / L	Permanent #:

INCLUSION CRITERIA

Check one		
YES	NO	Subject:
		1. Is ≥ 18 years ?
		2. Has signed informed consent?
		3. Is healthy as evidenced by responses on DCF 1 ?
		4. Has hands and wrists that are free of dermatoses, cuts, lesions, and other skin disorders ?
		5. Is willing to comply with all study protocol requirements ?

EXCLUSION CRITERIA

YES	NO	N/A	Subject:
			1. Is currently participating in another clinical study at this or any other facility ?
			2. Has participated in any type of hand or arm wash study within the past 7 days ?
			3. Has cuts, lesions, or other skin disorders on their hands or wrists ?
			4. Has soap, detergent, antibiotic and/or perfume allergies ?
			5. Has eczema or psoriasis on their hands or wrists ?
			6. Has used antibacterial/antimicrobial soaps, medicated lotions and creams and/or anti-dandruff shampoos within the last week?
			7. Has long (≥ 2 mm free edge) or artificial nails
Female	Female	Male	8. Is currently pregnant ? <input type="checkbox"/> Yes <input type="checkbox"/> No Of child-bearing potential: <input type="checkbox"/> Yes <input type="checkbox"/> No <input type="checkbox"/> Surgically Sterile <input type="checkbox"/> Post-menopausal If of child bearing potential - β -HCG Test Results: <input type="checkbox"/> negative <input type="checkbox"/> positive
Female	Female	Male	9. Is currently lactating?
			10. Has been medically diagnosed as having a medical condition such as: diabetes, hepatitis, an organ transplant, or AIDS (or HIV positive) ?
			11. Has another medical condition which in the opinion of the Investigator would preclude participation ?

Based upon dermatologic evaluation and the information contained in Data Collection 1 and 2, the subject is:

☐ **Qualified** ☐ **Not Qualified** for participation in this study.

Reasons for disqualification: _____

Interviewer's Signature	Date: ____ / ____ / ____ mm dd yy
Investigator's Signature::	Date: ____ / ____ / ____ mm dd yy

Data Collection Form 3

FOLLOW-UP VISIT

Study #	Hill Top Research, Inc.	Visit Code	Date	Subject Initials	Subject Screen #
01-108592-11 CRB-01-05-066-HB		Follow-up	mm / dd / yy	F / M / L	Permanent #:

Date Subject Entered the Study: mm / dd / yy	Follow-up Visit Date : mm / dd / yy
---	--

Does the subject's hands have the presence of pimples, blisters, or raised itching bumps surrounded by erythema and/or edema that may be indicative of a skin infection ?

YES NO If yes, complete below:

Clinical Observations: (Include date of onset and descriptions severity locations, etc.)

Comments:

Has the subject had any health related issues since the treatment procedure?

YES NO If yes, complete below:

Comments:

Investigator's Signature or designee	Date mm / dd / yy
--------------------------------------	----------------------

**Data Collection Form 4
MICROBIOLOGICAL RESULTS**

Study #	Hill Top Research, Inc.	Subject Initials	Permanent #
01-108592-11 CRB 01-04-051-HB		$\frac{\quad}{F} \quad \frac{\quad}{M} \quad \frac{\quad}{L}$	

BASE - Total # Organisms (CFU's) / mL of Sampling Solution						
PLATE	LEFT HAND DILUTIONS			RIGHT HAND DILUTIONS		
	10 ⁻⁴	10 ⁻⁵	10 ⁻⁶	10 ⁻⁴	10 ⁻⁵	10 ⁻⁶
1						
2						

TEST #1 (after first treatment) - Total # Organisms (CFU's) / mL of Sampling Solution										
PLATE	LEFT HAND DILUTIONS					RIGHT HAND DILUTIONS				
	10 ^{-1*}	10 ⁻²	10 ⁻³	10 ⁻⁴	10 ⁻⁵	10 ^{-1*}	10 ⁻²	10 ⁻³	10 ⁻⁴	10 ⁻⁵
1										
2										
3										

TEST #2 (after tenth treatment) - Total # Organisms (CFU's) / mL of Sampling Solution										
PLATE	LEFT HAND DILUTIONS					RIGHT HAND DILUTIONS				
	10 ^{-1*}	10 ⁻²	10 ⁻³	10 ⁻⁴	10 ⁻⁵	10 ^{-1*}	10 ⁻²	10 ⁻³	10 ⁻⁴	10 ⁻⁵
1										
2										
3										

Base Microbiologist(s):	Date:
Test Microbiologist(s):	Date:

*10⁻¹ = 1 mL of sampling solution spread across three plates.

Data Collection Form 5

ADVERSE EVENT

Study #	Hill Top Research, Inc.		Date	Subject Initials	Subject Screen #
01-108592-11 CRB-01-05-066-HB			mm / dd / yy	F / M / L	Permanent #:

Was reaction related to treatment? ☐ Not related ☐ Possibly related ☐ Definitely related ☐ Other (explain)

Did subject take any medication during the study period? ☐ YES ☐ NO If yes, complete section below.

Date of Onset: _____ Date Reported: _____ Date Resolved: _____

Describe event: _____

Action Taken: ☐ None ☐ Continued on study ☐ Withdrawn from the study ☐ Consulted physician

☐ Medication taken (Complete below) ☐ Hospitalized ☐ Other (explain)

Additional Comments:

FOLLOW - UP ACTION TAKEN

Date	Action Taken	Comments	Initials

CONCOMITANT MEDICATION TAKEN

Medication (Oral or Systemic)	Total Daily Dose	Start Date mm dd yy	Stop Date mm / dd / yy	Indication (Reason for Taking)
			/ /	
			/ /	
			/ /	

Investigator's Signature:

Recorded by:

Date

mm / dd / yy

Data Collection Form 6

PHYSICIAN'S ACTION REPORTING FORM

Study #	Hill Top Research, Inc.		Date	Subject Initials	Subject Screen #
01-108592-11 CRB-01-05-066-HB			mm dd / yy	F / M / L	Permanent #:

Date(s) of office visit(s): _____

Pertinent Medical History: (e.g., causes of similar reactions, known allergies,
potential involvement of current medications or medical conditions)

Test Product Exposure:

Use Began On: _____ Date Used Ended on: _____ Date Number of Uses: _____

Clinical Observations: (Include date of onset and descriptions: severity, locations, etc.)

Impression: _____

Treatment: _____

Follow Up: _____

Date Resolved: _____

Is condition related to use of the test products?

☐ Probably related* ☐ Not Related* ☐ Unknown

Reasons: _____

Physician's Signature

Date

Source Document 1
TREATMENT PHASE

Study #	Hill Top Research, Inc.		Permanent #'s
01-108592-11 CRB-01-05-066-IIB			

EVENT	TIME	PROCEDURE PERFORMED ACCORDING TO PROTOCOL?
Practice Wash	am/pm	<input type="checkbox"/> Yes / <input type="checkbox"/> No
Decontamination	am/pm	<input type="checkbox"/> Yes / <input type="checkbox"/> No
Base Contamination Procedure	am/pm	<input type="checkbox"/> Yes / <input type="checkbox"/> No
Base Bacterial Sampling Procedure	am/pm	<input type="checkbox"/> Yes / <input type="checkbox"/> No
Base Decontamination	am/pm	<input type="checkbox"/> Yes / <input type="checkbox"/> No
Test Contamination Procedure #1	am/pm	<input type="checkbox"/> Yes / <input type="checkbox"/> No
Test Product Treatment #1	am/pm	<input type="checkbox"/> Yes <input type="checkbox"/> No Water Temp: °F
Test Bacterial Sampling Procedure #1 (after first treatment)	am/pm	<input type="checkbox"/> Yes / <input type="checkbox"/> No
Test Contamination Procedure #10	am/pm	<input type="checkbox"/> Yes / <input type="checkbox"/> No
Test Product Treatment #10	am/pm	<input type="checkbox"/> Yes / <input type="checkbox"/> No Water Temp: °F
Test Bacterial Sampling Procedure #2 (after 10th treatment)	am/pm	<input type="checkbox"/> Yes / <input type="checkbox"/> No
Decontamination	am/pm	<input type="checkbox"/> Yes / <input type="checkbox"/> No Water Temp: °F

Recorder's Signature: _____	Date: ____/____/____
Reviewer's Signature: _____	Date: ____/____/____

Source Document 2
TREATMENT PHASE

Study #	Hill Top Research, Inc.	Permanent #'s
01-108592-11 CRB-01-05-066-HIB		

EVENT	TIME	PROCEDURE PERFORMED ACCORDING TO PROTOCOL?	
Test Contamination Procedure #2	am/pm	<input type="checkbox"/> Yes / <input type="checkbox"/> No	
Test Product Treatment #2	am/pm	<input type="checkbox"/> Yes / <input type="checkbox"/> No	Water Temp: °F
Test Contamination Procedure #3	am/pm	<input type="checkbox"/> Yes / <input type="checkbox"/> No	
Test Product Treatment #3	am/pm	<input type="checkbox"/> Yes / <input type="checkbox"/> No	Water Temp: °F
Test Contamination Procedure #4	am/pm	<input type="checkbox"/> Yes / <input type="checkbox"/> No	
Test Product Treatment #4	am/pm	<input type="checkbox"/> Yes / <input type="checkbox"/> No	Water Temp: °F
Test Contamination Procedure #5	am/pm	<input type="checkbox"/> Yes / <input type="checkbox"/> No	
Test Product Treatment #5	am/pm	<input type="checkbox"/> Yes / <input type="checkbox"/> No	Water Temp: °F
Test Contamination Procedure #6	am/pm	<input type="checkbox"/> Yes / <input type="checkbox"/> No	
Test Product Treatment #6	am/pm	<input type="checkbox"/> Yes / <input type="checkbox"/> No	Water Temp: °F
Test Contamination Procedure #7	am/pm	<input type="checkbox"/> Yes / <input type="checkbox"/> No	
Test Product Treatment #7	am/pm	<input type="checkbox"/> Yes / <input type="checkbox"/> No	Water Temp: °F
Test Contamination Procedure #8	am/pm	<input type="checkbox"/> Yes / <input type="checkbox"/> No	
Recorder's Signature:		Date: ____/____/____	
Reviewer's Signature:		Date: ____/____/____	

Source Document 2 (continued)

TREATMENT PHASE

Study #	Hill Top Research, Inc.			Permanent #'s
01-108592-11 CRB-01-05-066-HB				

Test Product Treatment #8	am/pm	<input type="checkbox"/> Yes / <input type="checkbox"/> No	Water Temp:	°F
Test Contamination Procedure #9	am/pm	<input type="checkbox"/> Yes / <input type="checkbox"/> No		
Test Product Treatment #9	am/pm	<input type="checkbox"/> Yes / <input type="checkbox"/> No	Water Temp:	°F
Recorder's Signature:		Date: / /		
Reviewer's Signature		Date: / /		

PILOT STUDY
RINSE OFF FORMULATION
SWH094-152, SW094-155

Clinical Study Report

CRA: Kathy Wiandt

Date: May 23, 2001

Study Statistician: Jeanne Philippo

Retention Limit: Until Superseded

Approved by: PBC 5/30/01

Subject: Results of Efficacy Evaluation of Two Handsoap Products in a Modified Healthcare Personnel Handwash Study Versus *Escherichia coli* – CRB-01-05-065-HB / HT# 01-108591-11.

Objective:

The objective of this study was to determine the ability of two antibacterial handsoap products to significantly reduce transient microbial flora (*Escherichia coli* 11229) on the hands after a single treatment and after ten (10) treatments.

Materials Tested:

Test Code	Test Material	Active Ingredient	Batch Number
A	Handwash Product	0% Salicylic Acid	SWH160-152
B	Handwash Product	2% Salicylic Acid	SWH160-155

Key Conclusions:

- After 1 wash, the 2.0% SA Hand Wash had a significantly higher reduction in log counts versus the test placebo (p-value=0.0043).
- After 10 washes, the 2.0% SA Hand Wash had a significantly higher reduction in log counts versus the test placebo (p-value=0.0022).

The summary of the mean logs recovered and the log reductions achieved following the first and tenth washes were determined.

Table I – Summary of HCPHWT Log ₁₀ Bacterial Results								
		Baseline	Log ₁₀ Counts – 1 Wash			Log ₁₀ Counts – 10 Washes		
Treatment	Sample Size	Mean	Mean	Change from Baseline	% Reduction	Mean	Change from Baseline	% Reduction
A-Handsoap 0% Salicylic Acid	6	7.63	5.21	2.42	99.6	5.44	2.19	99.4
B-Handsoap 2% Salicylic Acid	6	7.74	4.66	3.09	99.9	4.53	3.21	99.9

Study Summary:

Test Site: Hill Top Research, Miamiville, Ohio

Study Dates: May 8-14, 2001

Investigator: Gayle K. Mulberry, M.S.

Experimental Design: This was a randomized clinical study consisting of a one day test period and a single follow-up visit. Two test products were evaluated. Six subjects were used to evaluate each product.

Efficacy Measurements Taken: The subjects' hands were contaminated with a suspension of *E. coli*. Subjects' hands were contaminated eleven times and sampled three times using a plastic bag sampling procedure. The first contamination and sampling was for the determination of the base count. The second contamination and sampling was for determination of the test count after one treatment with the assigned Test Product. After eleven contamination steps and ten treatments with the assigned Test Products the hands were sampled using the plastic bag sampling procedure.

Subject Demographics: Twelve male and female subjects, ≥ 18 years old, who do not regularly use antibacterial/antimicrobial soaps, medicated lotions or creams and or antidandruff shampoos were enrolled into the study. Six subjects were used to evaluate one of two test products.

Overview: To become familiar with the wash procedure using a liquid hand soap, the subjects practiced the wipe procedure with Baby-san®. For the base count, subjects' hands were contaminated with *E. coli*. Immediately following the contamination step, the organisms on the subjects' hands were removed using a plastic bag sampling procedure.

Prior to each treatment wash, subjects' hands were contaminated with *E. coli*. After completing the contamination step, the subjects performed the test product application procedure with the assigned Test Product. The subjects lathered their hands for fifteen seconds and rinsed their hands for thirty seconds. Approximately five minutes following the wipe procedure, the organisms on both of the subjects' hands were removed using a plastic bag sampling procedure. Approximately five minutes following the tenth treatment, the organisms on the subjects' hands were removed using a plastic bag sampling procedure.

Samples of the subjects' sampling solutions were diluted, plated, and incubated. Following incubation, the numbers of colony forming units (CFU's) were enumerated. Antibacterial activity was determined by comparing the number of bacteria removed from the hands after one treatment with the assigned Test Product and ten treatments with the assigned Test Product to the number of bacteria removed from unwashed hands.

Data Analysis:

For the bag juice results, each subject's base sampling CFU's was compared to their test sampling CFU's using a nonparametric Wilcoxon paired signed-rank test. P-values ≤ 0.10 were considered statistically significant. Percent change for each organism was computed by the following formula:

$$1 - \left(\frac{\text{geometric mean of the test CFU's}}{\text{geometric mean of the baseline CFU's}} \right) \times 100$$

Treatment comparisons were analyzed by a Wilcoxon-Mann-Whitney Test using Exact methods.

Regulatory/Ethics Status:

This study was conducted in compliance with federal, state, and local regulations, guidelines, and standards including those related to Informed Consent and Good Clinical Practices as specified under 21 CFR 321.66.

Subject Accountability:

Twenty subjects were screened for the study. Twelve (12) subjects were screened, enrolled and completed this study. Five subjects met the study qualifications, but were excluded because they were extra subjects.

Two subjects were excluded because of open cuts on their hands. One subject was excluded because they were allergic to penicillin.

Adverse Events:

There were no adverse events in this study.

W. W. W. W. W.

Clinical Research Associate

James C. Phillips

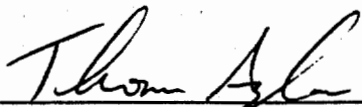
Statistician

HTR Study No.: 01-108591-11
Sponsor Study No.: CRB-01-05-065-HB

QUALITY ASSURANCE STATEMENT

This study was inspected in accordance with the Standard Operating Procedures of Hill Top Research, Inc. To assure compliance with the study protocol, the Quality Assurance Unit performed an inspection during the conduct of this study and completed an audit of the study records.

Data reviewed by:

	5-17-01
Thomas Asplan, A.A.S, B.S.	Date
Auditor, Quality Assurance	

CLINICAL STUDY PROTOCOL

Clinical Research & Biometrics Department
Sharon Woods Technical Center
Cincinnati, Ohio 45241

Title: Efficacy Evaluation Of Two Liquid Soap Products In A Modified Health Care Personnel Handwash Study Versus *Escherichia Coli*

Study Number: CRB-01-05-065-HB HT# 01-108591-11

Issue Date: 5/4/01

Products Tested: Antibacterial Handsoap Prototype
Antibacterial Handsoap Prototype

Test Facility: Hill Top Research, Inc.
Main and Mill Streets
Miamiville, Ohio 45147

Microbiology Samples: The Procter and Gamble Company
Miami Valley Laboratories

Principal Investigator: Gayle Mulberry, M.S.

Sub-Investigators: Kathleen A. Baxter, B.S.
Ann R. Brady, A.S.

Test Sponsor: The Procter & Gamble Co., Inc.
Sharon Woods Technical Center
11520 Reed Hartman Highway
Cincinnati, Ohio 45241

		<u>work</u>	<u>home</u>
Sponsor Toxicologist:	Candace Doepker, Ph.D	(513) 626-5536	
Sponsor Representative/CRA:	Kathy Wiandt, B.S.	(513) 626-5225	(513) 398-6035
Sponsor Statistician:	Jeanne Philippo, B.A.	(513) 626-5937	
Expected Study Start Date:	May 8, 2001		
Expected Study End Date:	May 14, 2001		

I. Study Objective and Background

A. Objective

The objective of the study is to determine the ability of a two antibacterial handwash product containing to significantly reduce transient microbial flora (*Escherichia coli* 11229) on the hands after a single treatment and after ten (10) treatments.

B. Background

The skin microflora can be divided into two (2) groups, the resident flora and the transient flora. The resident flora includes organisms that are consistently present on the skin. The transient flora are the contaminating skin organisms resulting from contact with the environment. They comprise a wide variety of Gram positive and Gram negative species that can be responsible for the spread of infections and gastrointestinal diseases.

Since the benefits that result from washing with antibacterial soaps can not be easily measured under consumer use conditions, it is necessary to do controlled clinical studies to demonstrate their efficacy. This clinical study is a modification of an ASTM test method, "Evaluation of Health Care Personnel Handwash Formulation"⁽¹⁾ and reported in the Tentative Final Monograph for Health Care Antiseptic Drug Products⁽²⁾. It is used to determine the ability of an antimicrobial handwashing agent, when used in a hand washing procedure, to reduce the transient microbial flora (contaminants). This study is designed to demonstrate the efficacy of two liquid handsoaps in reducing the numbers of a marker organism, *Escherichia coli* ATCC 11229 on the hands after a contamination and a single handwash and after ten handwashes. Efficacy is determined by comparing the numbers of marker organisms on the hands before and after using the test products.

C. Study Safety Statement

This testing meets the ethical requirements stipulated in the Sponsor's Policy for Research Involving Human Subjects. Appropriate safety testing has been completed and risk assessments justify the placement of the test products in this study at these concentrations (levels of exposure).

II. Study Summary

A. Overview

This randomized clinical study will consist of a one day test period and a follow-up visit. Two (2) test products will be evaluated. Twelve (12) male and female subjects, ≥ 18 years old, who do not regularly use antibacterial/antimicrobial soaps, medicated lotions and creams, and or antidandruff shampoos (Appendix E), will be enrolled into the study. Six (6) subjects will be used to evaluate each test product.

On the day of the study, the subjects will report to the clinical test facility. During this period, subjects' hands will be contaminated with a suspension of *E.coli*. Subjects' hands will be contaminated eleven (11) times and sampled three (3) times using a plastic bag sampling procedure. The first contamination and sampling will be for the determination of the base count. The second contamination and sampling will be for determination of the test count after one (1) treatment with the assigned Test Product. After eleven (11) contamination steps and ten (10) treatments with the assigned Test Products the hands will be sampled using the plastic bag sampling procedure.

To become familiar with the wash procedure using the liquid hand soap, the subjects will begin the test procedure by first performing a practice wash with Baby-san®. For the base count, subjects will have their hands contaminated with *E coli*. Immediately following the contamination step, the organisms on the subjects' hands will be removed using a plastic bag sampling procedure.

Prior to each treatment wash, subjects' hands will be contaminated with *E. coli*. After completing the contamination step, the subjects will perform the test product application procedure with the assigned

Test Product. Approximately five (5) minutes following the first procedure, the organisms on both of the subjects' hands will be removed using a plastic bag sampling procedure. Approximately five (5) minutes following the tenth treatment, the organisms on the subjects' hands will be removed using a plastic bag sampling procedure.

Aliquots of the subjects' sampling solutions will be diluted, plated, and incubated. Following incubation, the number of colony forming units (CFU's) will be enumerated. Antibacterial activity is determined by comparing the number of bacteria removed from the hands after one (1) treatment with the assigned Test Product and ten (10) treatments with the assigned Test Product to the number of bacteria removed from unwashed hands.

B. Study Schedule

1. Subject Qualification and Enrollment

Prospective subjects will visit the test facility to be screened for their eligibility to participate in the study. Eligibility will be based upon information provided in the Demographics/Dermatological/Medical History Form (DCF 1) and the Inclusion Exclusion Form (DCF 2); and completion of a written informed consent (Appendix A).

2. Test Period

Subjects continuing on the study will be assigned a permanent subject number. Subjects will be assigned to one of the two test products according to the study randomization.

The following outlines the schedule of procedures for the test day:

1. Subjects will perform a practice wash with Baby-san® (Appendix D).
2. Subjects will rinse their hands with 70% alcohol and rinse their hands under running tap water (Section G).
3. Subjects' hands will be contaminated (Section E).
4. Subjects' hands will be sampled for a base count (Section F).
5. Subjects will rinse their hands with water for 30 seconds (Section G).
6. Subjects will rinse their hands with 70% alcohol and rinse with tap water (Section G).
7. Subjects' hands will be contaminated (Section E).
8. Subjects will wash their hands following the wash procedure for the assigned Test Product (Section C, Appendix C).
9. Subjects' right and left hands will be sampled for a treatment value four (4) minutes and thirty (30) seconds \pm thirty (30) seconds after the first wash with the assigned Test Product (Section F).
10. The hands will be rinsed for thirty seconds.
11. Subjects will perform steps 7 and 8 (above) a total of nine (9) more times at a minimum of five (5) minutes between each wash procedure.
12. The subjects' hands will be sampled for a treatment value four (4) minutes and thirty (30) seconds \pm thirty (30) seconds after the tenth wash with the assigned Test Product (Section F).
13. Subjects' hands will be disinfected with a bland soap and water wash and Hibiclens® (4% chlorhexidine gluconate) wash and with a 70% alcohol rinse (Section G).

Note: A detailed schedule of the above procedures can be found in Appendix D.

To ensure that any delayed adverse events, such as primary skin infections, are reported to the Study Investigator, all test subjects will be given a copy of Subjects' Instructions Following Study

Completion (Appendix B) before leaving the clinical site after they have completed the study. This sheet will instruct the subjects to examine their hands and wrists daily until the final scheduled visit for the presence of pimples, blisters, or raised, red itching bumps surrounded by erythema and/or edema that may be indicative of a skin infection. Subjects, who notice such lesions, will be instructed to call the clinical test site. The subjects will return to the clinical test site within four (4) to nine (9) days after the study procedures have been completed to have their hands and wrists examined by a technician. The technician will complete DCF 3 for each subject on their follow-up visit.

C. Product Treatment Procedure

Subjects will wash their hands and wrists according to the procedure described in Product Treatment Procedure, Appendix C. In general the following should be noted:

- a. Water temperature should be closely monitored and maintained at 95-100°F. The water temperature should be recorded on Source Document 1 or 2 before each wash.
- b. Water pressure should be adjusted to a flow of 4 L/minute. This may be accomplished by placing a 2000 mL glass beaker or flask under each spigot to be used for subjects' hand washing. Allow the water to flow into the beaker. Adjust the water flow at each spigot accordingly, so that the beaker fills within thirty (30) seconds.
- c. Subjects are to be closely supervised as they lather and wash their hands and wrists. The washes will be recorded on Source Documents 1 or 2.

D. Preparation of Bacterial Suspensions

A stock culture of *Escherichia coli*, ATCC 11229, will be prepared by transferring at least isolated (3) colony from an agar plate or slant aseptically to a tube containing sterile Trypticase Soy Broth (TSB). The inoculated broth will then be incubated for 24 ± 4 hours at $35 \pm 2^\circ \text{C}$. At least three (3) additional 24 hour broth transfers will be made in tubes containing appropriate volumes TSB from this broth culture.

A 2-liter flask containing 1000 mL of TSB will be inoculated with 1.0 mL of the final 24 hour broth transfer. The flask will be incubated for 24 ± 4 hours at $35 \pm 2^\circ \text{C}$. Prior to any withdrawal of culture, whether for hand contamination or for numbers assay, the suspension will be stirred or shaken. The suspension will be assayed for number of organisms at the beginning and end of the treatment period. A suspension will not be used for more than eight (8) hours.

E. Contamination

Note: Prior to contamination, subjects hands must be visibly dry. Also, care should be taken to ensure that the culture is evenly spread over both hands

A total volume of 4.5 mL of the assigned bacterial suspension will be dispensed into the subjects' cupped hands in 1.5 mL increments. After each 1.5 mL aliquot is added, the suspension will be rubbed thoroughly over the surface of both hands, not going above the wrist and avoiding the nail beds. Each application and spreading should last approximately twenty (20) seconds. Between each aliquot the hands will be held away from the body and allowed to air dry for approximately thirty (30) seconds. Following the third 1.5 mL aliquot, the hands are allowed to air dry for approximately one (1) minute. A record of base and test contaminations will be documented on Source Document 1 or 2.

F. Bacterial Sampling Procedure

For removal of bacteria from the subjects' hands, loose fitting plastic bags with low bioburden will be placed on each subject's right and or left hands. A 75 mL aliquot of stripping solution [0.1% Triton X-100 in 0.075 M phosphate buffer, 1.0% polysorbate (Tween) 80, 0.3 % Lecithin, pH 7.9] will be aseptically added into each bag. The same solution will be used for the base counts and test counts.

The bag on each hand will be secured at the wrist with a child's size tourniquet and massaged for one (1) minute in a uniform manner by a lab technician. Aliquots of the solution will be aseptically obtained directly from the bag without touching the hands in the process and will be appropriately diluted in a sterile diluent with the appropriate neutralizer (for the test wash samples only). A record of base and test samplings will be documented on Source Document 1.

The solution samples for bacteria counts will be labeled by either an Investigator derived code or the actual subject's number so that the individuals who prepare the plates and count the CFU's are unaware of the sources of the sampling solution.

The solution will be aseptically placed in a sterile test tube. The test tube will be affixed with the subject number, baseline or post-treatment, and placed on ice for microbiological analysis. The sponsor will analyze the samples for microbiological content. The transfer of the microbial specimens will be recorded on Source Document 3.

G. Disinfection of Hands

After the baseline sampling, the subjects will rinse their hands for thirty (30) seconds under running tap water. The subjects' hands will be disinfected with a 70% alcohol wash. Subjects' hands will be squirted with 70% alcohol for approximately ten (10) seconds. Subjects will rub the alcohol over the surface of their hands and wrists for approximately fifteen (15) seconds. Subjects will rinse their hands and wrists under running tap water for approximately fifteen (15) seconds and dry their hands and wrists with paper towels.

After the final sampling is completed, the subject's hands will be washed with a bland soap (provided by the investigator) for approximately thirty (30) seconds and rinsed for approximately fifteen (15) seconds. The subjects' hand will then be washed with Hibiclens® (4% chlorhexidine gluconate) for at least sixty (60) seconds. Subjects' hands and wrists will be rinsed with a 70% alcohol wash for ten (10) seconds. The subjects will rub the alcohol on all surfaces of their hands for fifteen (15) seconds and allow their hands to air dry.

A record of each disinfection procedure will be recorded on Source Document 1.

H. Plating and Incubation of the Organisms

Baseline specimens will be serially diluted in half-strength (0.0375 M) buffer (without Trition X-100) in ten-fold dilutions to 10^{-1} , 10^{-2} and 10^{-3} . The diluted specimens will be plated using an automated plating system (Eddyjet system) onto MacConkey's agar. Post treatment specimens will be serially diluted in ten-fold dilutions to 10^{-1} , 10^{-2} , 10^{-3} and 10^{-4} . Using an automated plating system (Eddyjet system), the undiluted and diluted specimens will be plated onto MacConkey's agar. The media for these analyses are shown in Appendix F.

Plated samples will be incubated aerobically for 18 - 24 hours at $35 \pm 2^{\circ}\text{C}$. The plates will be analyzed using the Counterstat®. The results will be reported as colonies per mL using the Counterstat® software package.

The results will be recorded in an electronic file created by the sponsor.

III. Study Population

Subjects will be screened for their eligibility to participate based upon information provided in the Demographics/Dermatological/Medical History Form [Data collection form (DCF) 1]. Only subjects meeting the inclusion/exclusion criteria, outlined in DCF 2, will be allowed to participate in the study. If a subject is admitted to this study in apparent violation of any of the above criteria, the reason(s) for admission will be noted by the Investigator or her designee.

A. Subject Inclusion Criteria

Subjects will be eligible for enrollment if they:

1. Are a male or female, over 18 years of age ;
2. Have signed a written informed consent (Appendix A);
3. Are in good health, as evidenced by response to the Demographics/Dermatological/ Medical History Form (DCF 1);
4. Have hands and wrists that are free of dermatoses, cuts, lesions, and other skin disorders;
5. Are willing to comply with all study protocol requirements.

B. Subject Exclusion Criteria

Subjects will not be enrolled in the study if they:

1. Are currently participating in another clinical study at this or any other facility;
2. Have participated in any type of arm or hand wash study within the past 7 days;
3. Have cuts, lesions, or other skin disorders on their hands or wrists;
4. Have soap, detergent, antibiotic, and or perfume allergies;
5. Have eczema or psoriasis on their hands or arms;
6. Are using antibacterial/antimicrobial soaps (liquids and/or bars), medicated lotions and creams, and/or anti-dandruff shampoos in the home within the last week (Appendix E);
7. Have excessively long or artificial nails (≥ 2 mm free edge) which would interfere with sampling;
8. Are currently pregnant;
9. Are currently lactating;
10. Have been diagnosed as having a medical condition which would preclude participation such as: diabetes, hepatitis, an organ transplant, or AIDS (or HIV positive); and/or
11. Have any other medical condition, which in the opinion of the Investigator, would preclude participation.

D. Subject Number Assignment and Randomization

Upon entry into the study, each subject will be assigned a screening number beginning with 1001. Subjects will be assigned a permanent consecutive number, beginning with 001, as they are accepted into the study. This number will be used to identify the subject for the duration of the study.

IV. Study Material

A. Test Product

The test products will be sent by the Sponsor to the clinical site prior to study initiation. The test products will be identified with the appropriate label affixed to the outside of each container.

B. Shipping of Treatment Products and Other Study Supplies

The quantity of all treatment products and other study supplies, shipped to and returned from the clinical site, will be documented by the test site. The treatment products will be packed into one or more cartons labeled with:

1. the study number;
2. distributor statement (i.e., "Distributed by Hill Top Research, Inc." with the facility's full

address and phone number);

3. any applicable safety and handling procedures.

C. Return of Study Materials

Upon completion of the study, the Investigator(s) will insure that all test products and study materials, whether completely used, partially used, or unused will be returned to the Sponsor at the following address:

The Procter & Gamble Company
Sharon Woods Technical Center
11520 Reed Hartman Highway
Cincinnati, Ohio 45241
Attn.: Kathy Wiandt

V. Other Study Documentation

A. Adverse Events

Should any unexpected or serious adverse event occur during the clinical study or as a result of test product or study procedures, the subject will be requested to return to the site to be examined by the investigator or designee. The Investigator will determine if the adverse event is likely to be associated with product treatment or the study procedures. The investigator or other qualified medical personnel will determine if the event warrants termination of participation and/or to prescribe treatment, if necessary. The Investigator will notify the Sponsor representatives, Ward L. Billhimer, 513-626-1926 (work) or 513-831-8163 (home) or Kathy Wiandt, 513-626-5225 (work) or 513-398-6035 (home).

Each subject will need to be followed until the resolution of any adverse event. Information pertaining to the presenting signs, working diagnosis, assessment of the relationship of the adverse event to the product treatment, results of the follow-up visits and any prescribed treatment, will be documented in DCF 4. If treatment by a physician is necessary, this treatment will be documented on DCF 5.

The following criteria will be used to determine the reporting time frame.

1. Any serious adverse events or adverse events requiring immediate medical attention will be reported to the Sponsor's Monitor immediately (night or day) by telephone.
2. Adverse events resulting in subject termination from the study will be reported during the immediate business day by telephone.
3. Adverse events that do not require discontinuation of test participation can be reported during the immediate business day or next business day by telephone.
4. In the event of a serious adverse reaction, not necessarily related to use of the test product, or in the event of a death from any cause, the Investigator must report the event to the Sponsor's Monitor.

B. Protocol Amendments

If it becomes necessary to modify this protocol, the modification will be documented by a protocol amendment signed by the investigator and a representative of the Sponsor. All amendments to the final protocol will be consecutively numbered and will describe any changes made and the rationale for making the changes.

C. Protocol Deviations

If a deviation from the final protocol occurs, it is the responsibility of the Investigator, or designee, to notify the Clinical Research Associate or designee. The deviation and subsequent notification will be documented appropriately.

D. Study Monitoring

The Investigator will permit a representative of the Sponsor (usually the Clinical Research Associate) to visit the facility during the course of the study to monitor study progress. During the visit(s), the Investigator will permit the monitor to inspect all forms and corresponding study subject's records to verify adherence to the protocol. The study monitor will also be permitted to review and verify test articles, wash procedure, and any Investigator-generated or Sponsor-generated study documents. The monitor will document and discuss this visit with the Investigator, or his designee, including any problems that are to be resolved.

VI. Statistical Analyses

The sponsor will be responsible for all statistical analyses. For the bag juice results, each subject's base sampling CFU's will be compared to their test sampling CFU's using a nonparametric Wilcoxon paired signed-rank test. P-values ≤ 0.10 will be considered statistically significant. Percent change for each organism will be computed, if needed, by the following formula:

$$1 - \frac{(\text{geometric mean of the test CFU's})}{\text{geometric mean of the baseline CFU's}} \times 100$$

VII. Investigator Responsibilities

A. Subject Informed Consent

All subjects will be informed as to the type of study, the general nature of the products being tested, and any known or anticipated adverse reactions which might result from participation. Each subject must provide the Investigator with written informed consent to serve as a participant in the study. Basic elements of informed consent are outlined in 21 CFR 50.25.

B. Final Report

The Sponsor will generate a final report of clinical results. The investigator will provide a detailed description of the adverse events and deviations from the protocol. The investigator will also include an accounting of the subjects screened, eliminated, enrolled and terminated. The Investigator will submit the legible copies of all data collection forms. The Sponsor may request one (1) copy of all case report forms before the Investigator's report is ready for submission to the Sponsor.

C. Record Retention

The Investigator will retain all study records in accordance with the test facility's SOP's.

D. Confidentiality

The Investigator and employees of the test facility are obligated to keep any information confidential regarding any of the personal cleansing products and all aspects of the study, as subject to the terms and conditions of the Laboratory Services Agreement between the test facility and Sponsor.

VIII. References

1. *Annual Book of ASTM Standards*, Volume 11.04, ASTM Designation: E 1174-94, Standard Test Method for "Evaluation of Health Care Personnel Handwash Formulation".
2. Tentative Final Monograph for Health-Care Antiseptic Drug Products; Proposed Rule, 21 CFR Parts 333 and 369, *Federal Register*, Volume 59, No. 116, June 17, 1994.

IX. Attachments

The following Appendices, Data collection forms are included as attachments to the Final Protocol:

- A Written Informed Consent
- B Subject's Follow-up Instructions
- C Product Treatment
- D Schedule of Test Period Procedures
- E List of Representative Antibacterial/Antimicrobial Products
- F Microbiological Media and Methods

Data Collection Forms

- 1 Demographics/Dermatological Medical History Form
- 2 Inclusion/Exclusion Form
- 3 Follow- up Visit
- 4 Adverse Event
- 5 Physician's Report Form

Source Documents

- 1 Treatment Phase (Baseline, Wash 1 and Wash 10)
- 2 Treatment Phase (Washes 2 through 9)
- 3 Shipping of Microbiological Specimens

X. Sponsor and Investigator Concurrence

For The Procter and Gamble Company

PREPARED BY:

Kathy Wiandt
Kathy Wiandt, B.A., Clinical Research Associate
Clinical Research and Biometrics Department

Date: 5/4/01

STATISTICIAN:

Jeanne C. Philippo
Jeanne C. Philippo, B.A., Statistician
Clinical Research and Biometrics Department

Date: 5/4/01


APPROVED BY:

Ward L. Billhimer
Ward L. Billhimer, M.S., Senior Scientist
Clinical Research and Biometrics Department


Date: 5/4/01

Agreed and Accepted by Hill Top Research, Inc. and the Study Investigator(s) for
CRB-01-05-064-HB:

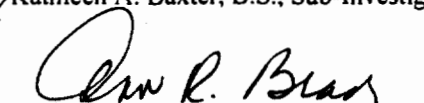
I certify that I have reviewed and approved the protocol, informed consent form, and other associated documents and agree to abide by their terms. In addition, I agree to conduct this clinical study in compliance with federal, state and local government regulations, guidelines and standards applicable to such studies.


Gayle K. Mulberry, M.S., Investigator

Date: 5-7-01


Kathleen A. Baxter, B.S., Sub-Investigator

Date: 5.07.01


Ann R. Brady, A.S., Sub-Investigator

Date: 5.7.01

Appendix A
HT-01-108591-11
CRB-01-05-065-HB

WRITTEN INFORMED CONSENT

To be provided by the clinical site.

Appendix B
HT-01-108591-11
CRB-01-05-065-HB

SUBJECT'S INSTRUCTIONS FOLLOWING STUDY COMPLETION

You have just completed participation in a clinical study, "Efficacy Evaluation Of Two Liquid Soap Products In A Modified Health Care Personnel Handwash Study Versus *Escherichia Coli*". During this study, a quantity of bacteria (*E. coli*) was placed on the surface of both your hands. Although we do not expect you to have any adverse experience as a result of participation in this study, there is a remote possibility that an infection may develop on your hands and wrists within four (4) to nine (9) days.

To determine whether you have developed an infection from the test bacteria, we would like you to examine your hands and wrists daily. If you notice the appearance of any pimples, blisters or raised bumps surrounded by redness and/or swelling, please contact Gayle Mulberry or Ann Brady at (513) 831-3114 during normal business hours (8:00 am-5 p.m.) or at (513) 831-3354 after hours.

You are required to return to the test site for a follow-up visit. Your follow-up is scheduled for:

Date

Time

Thank you for your cooperation.

Appendix C

HT-01-108591-11
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PRODUCT TREATMENT PROCEDURE

- Water temperature should be maintained at 95 - 100° F.
- The temperature should be checked and recorded before each wash.
- The water pressure at each spigot to be used for the study should flow at 4 L/min.
- Subjects should remove all jewelry from hands and wrists prior to start of wash procedure.

The following wash procedure will be performed by each subject:

1. Subjects will be instructed to wet their hands under the running water.
2. **2.0 mL of product** will be dispensed from a disposable syringe into the subjects' hands by a laboratory technician.
3. The technician will instruct the Subjects to lather all surfaces of their hands and wrists for **fifteen (15) seconds**.
4. Subjects will rinse their hands under running tap water for thirty (30) seconds.
5. A. For test washes #1 and #10, hands will not be dried.

B. For test washes #2 through #9, subjects will dry their hands with paper towels.

(Note: following the practice wash, subjects' hands will be disinfected and contaminated.)

6. Bags will be placed on the subjects' right and left hands for sampling after the first wash and after the tenth treatment. Sampling time will be approximately five (5) minutes following the wash with the test product.

Appendix D
HT-01-108591-11
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SCHEDULE OF TEST PERIOD PROCEDURES

- 1. Practice treatment with Test Product:**
 - subjects wet hands under running tap water
 - dispense 2.0 mL of Baby San® into subjects' hands
 - subjects lather hands and wrists for fifteen (15) seconds
 - subjects rinse hands under running tap water for thirty (30) seconds
 - subjects dry hands with a paper towel
- 2. 70% alcohol rinse**
 - squirt backs and palms of subjects' hands with 70% alcohol for 10 seconds
 - subjects rub alcohol over hands for 15 seconds
 - subjects rinse hands under running tap water for 15 seconds
 - subjects dry hands with paper towels
- 3. Base contamination**
 - dispense 1.5 mL aliquot of bacterial suspension onto both subjects' hands
 - subjects rub aliquot over hands for 20 seconds
 - allow subjects' hands to air dry for approximately 30 seconds
 - repeat application 2 times
 - allow subjects' hands to air dry 1 minute after the last application
- 4. Base sampling**
 - place bags on subject's right and left hands
 - dispense 75 mL stripping solution into each bag
 - secure bags
 - massage for 1 minute
 - sample each bag
- 5. Water rinse**
 - subjects rinse hands with water for 30 seconds
- 6. 70% alcohol rinse**
 - perform as above
- 7. Test contamination (prior to Test Product treatments 1 through 10)**
 - perform as above under base contamination
- 8. Test Products Treatments (treatments 1 through 10)**
 - perform as described under practice treatment
 - for treatments #1 and #10, hands will not be dried prior to sampling
 - for treatments # 2 through #9 subjects will dry hands with paper towels
- 9. Test sampling - Following Treatment 1**
 - perform as above under base sampling
 - subjects rinse hands with water for 30 seconds after the first test sampling

Appendix D (continued)

HT-01-108591-11

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10. Test sampling - Following Treatment 10

- place bag on of the subject's hands
- dispense 75 mL stripping solution into the bag
- secure bag
- massage for 1 minute
- sample bag

11. Disinfection

- subject rinse hands for thirty (30) seconds
- squirt subjects' hands with 2 mL of bland soap
- subjects wash hands and wrists for approximately 30 seconds
- subjects rinse hands and wrists for approximately 15 seconds
- squirt subjects' hands with 5 mL of Hibiclens®
- subjects wash hands and wrists for at least 60 seconds
- subjects rinse hands and wrists for 15 seconds
- squirt backs, palms and wrists of subjects' hands with 70% alcohol for 10 seconds
- subjects rub alcohol over hands and wrists for 15 seconds
- subjects' hands will be allowed to air dry

Appendix E
HT-01-108591-11
CRB-01-05-065-HB

LIST OF ANTIBACTERIAL / ANTIMICROBIAL PRODUCTS

Medicated Acne Cleansers

Benzac W Wash 5
Desuam-X 5 Wash
Benzac W Wash 10
Desquam-X 10m Wash
Fostex 10% BPO Wash
Oxy 10 Wash
Propa P.H. Liquid Acne Soap
PanOxyl 5
Fostex 10% BPO
PanOxyl 10
Clearasil Antibacterial Soap
Sastid Plain Therapeutic Shampoo and Acne Wash
Oxy Clean Soap
Fostex Medicated Cleansing Bar
Salicylic Acid and Sulfur Soap
Sulfur Soap

Antidandruff Shampoos

Head and Shoulders (all formulas)
Selsun Blue (all formulas)
Pert Plus for Dandruff
Suave for dandruff
Neutrogena T-gel
Neutrogena T-sal
Scalpacin
Tegrin
Any antidandruff shampoo

Anti-bacterial Soaps

Safeguard bar and liquid
Lever 2000 bar and liquid
Irish Spring bar
Dial bar and liquid
Softsoap Antibacterial Soap

Antibiotic Ointments and Creams

Bacitracin
Polysporin
J & J First Aid Cream
Neomycin

Antibacterial Dishwashing Liquids

Dawn
Joy
Dial
Palmolive

Appendix F
HT-01-108591-11
CRB-01-05-065-HB

MICROBIOLOGICAL MEDIA AND METHODS

0.075M Phosphate Buffer Solution with Neutralizers

Weigh 0.4 grams of KH_2PO_4 , 10.1 grams of Na_2HPO_4 , 10.0 grams of Polysorbate (Tween) 80, 3 grams of lecithin, and 1.0 gram of Triton X-100. Dissolve in 1 liter of distilled or deionized water. Adjust to pH 7.9 \pm 0.1 with 1 N HCl or 1 N NaOH. Dispense buffer in bottles so that after autoclaving the volume equals 75 \pm 1 mL. Loosely cap bottles and sterilize in the autoclave at 121°C.

0.0375M Phosphate Buffer Solution with Neutralizers

Weigh 0.2 grams of KH_2PO_4 , 5.05 grams of Na_2HPO_4 , 10.0 grams of Polysorbate (Tween) 80 and 3 grams of lecithin. Dissolve in 1 liter of distilled or deionized water. Adjust to pH 7.9 \pm 0.1 with 1 N HCl or 1 N NaOH. Dispense buffer in appropriate volumes. Loosely cap vessels and sterilize in the autoclave at 121°C.

MacConkey's Agar

Suspend 50 grams in 1 liter of distilled or deionized water. Loosely cap flask and sterilize in the autoclave at 121°C. Cool to 45-50°C in a water bath. Pour in sterile 15 x 100 mm Petri dishes. Allow to cool and solidify on a level flat surface. Check for sterility. Prepared plates are stored at 2 - 8°C and used within 30 days.

Estimated Plate Count Procedure

Do not record counts on crowded plates from the highest dilution as too numerous to count (TNTC). If the number of colonies per plate exceeds 250, count colonies in those portions of the plate that are representative of colony distribution and calculate the Estimated Standard Plate Count (ESPC) from these counts. The ESPC will be determined utilizing the grid embossed area on the lighted surface of the colony counter. Each large square on the grid is 1 cm². If there are fewer than 10 colonies per square centimeter, count colonies in 12 squares, selecting, if representative, six consecutive squares horizontally across the plate and six consecutive squares at right angles, being careful not to count a square more than once. When there are more than 10 colonies per square centimeter, count colonies in four such representative portions. In both instances, multiply the average found per square centimeter by the area of the plate used to determine the estimated number of colonies per plate.

If the total number of CFU's have been estimated according to the procedure described above, ESPC (Estimated Standard Plate Count) should be recorded following the value.

Note: If the highest dilution plated contains >250 CFU's and a count \leq 300 CFU's has been previously determined, that value may be reported. It will not be necessary to estimate the total CFU's on a plate containing >250 CFU's using the above procedure. Plates containing the highest dilution of test specimen plated and the CFU counts are greater than 300, then the above procedure should be used to determine the total CFU count.

Data Collection Form 1
DEMOGRAPHICS/DERMATOLOGICAL/MEDICAL HISTORY FORM

Study #	Hill Top Research, Inc.	Visit Code	Date	Subject Initials	Subject Screen #
01-108591-11 CRB-01-05-065-HB		Subject Qualification	mm / dd / yy	F / M / L	Permanent #:

Gender: <input type="checkbox"/> Male <input type="checkbox"/> Female	Age: _____ Years
--	-------------------------

Does the subject have any of the following at the treatment sites?

I. DERMATOLOGIC DISORDER	No	Yes	Don't Know
1. Psoriasis ?			
2. Eczema ?			
3. Skin Cancer ?			
4. Skin Allergies ? Please specify:			
5. Hives ?			

Does the Subject have any of the following (present and past)?

II. OTHER MEDICAL INFORMATION	No	Yes	Don't Know
1. Allergies.? Please specify.			
2. Hepatitis ?			
3. Heart and Vascular Disease?			
4. Liver Disease ?			
5. Kidney Disease ?			
6. Tuberculosis ?			
7. Diabetes ? Controlled? Diet [] Oral [] Insulin []			
8. Cancer ?			
9. Auto-immune disease (Lupus erythematosus, thyroiditis, AIDS, etc.) ?			
10. Organ transplant ?			
11. Any other condition not listed ? Please specify:			

Is the subject taking any medication? If yes, please specify below:

III. MEDICATION	No	Yes	Don't Know
1. Antibiotics, oral or systemic ?			
2. Cortisone, Steroids, ACTH, Anti-reaction Drugs ?			
3. Heart Medication ?			
4. Insulin ?			
5. Other ?			

Comments:

Based on the above medical history, the subject is: ☐ **Qualified** or ☐ **Not qualified** for the study.

Interviewer's Signature:	Date: _____ / _____ / _____ mm dd yy
---------------------------------	--

Data Collection Form 2
INCLUSION / EXCLUSION FORM

Study #	Hill Top Research, Inc.	Visit Code	Date	Subject Initials	Subject Screen #
01-108591-11 CRB-01-05-065-HB		Subject Qualification	mm / dd / yy	F / M / L	Permanent #:
INCLUSION CRITERIA					
Check one					
YES	NO	Subject:			
		1. Is ≥ 18 years ?			
		2. Has signed informed consent?			
		3. Is healthy as evidenced by responses on DCF 1 ?			
		4. Has hands and wrists that are free of dermatoses, cuts, lesions, and other skin disorders ?			
		5. Is willing to comply with all study protocol requirements ?			
EXCLUSION CRITERIA					
YES	NO	N/A	Subject:		
			1. Is currently participating in another clinical study at this or any other facility ?		
			2. Has participated in any type of hand or arm wash study within the past 7 days ?		
			3. Has cuts, lesions, or other skin disorders on their hands or wrists ?		
			4. Has soap, detergent, antibiotic and or perfume allergies ?		
			5. Has eczema or psoriasis on their hands or wrists ?		
			6. Has used antibacterial/antimicrobial soaps, medicated lotions and creams and/or anti-dandruff shampoos within the last week?		
			7. Has long (≥ 2 mm free edge) or artificial nails		
Female	Female	Male	8. Is currently pregnant ? <input type="checkbox"/> Yes <input type="checkbox"/> No Of child-bearing potential: <input type="checkbox"/> Yes <input type="checkbox"/> No <input type="checkbox"/> Surgically Sterile <input type="checkbox"/> Post-menopausal If of child bearing potential - β -HCG Test Results: <input type="checkbox"/> negative <input type="checkbox"/> positive		
Female	Female	Male	9. Is currently lactating?		
			10. Has been medically diagnosed as having a medical condition such as: diabetes, hepatitis, an organ transplant, or AIDS (or HIV positive) ?		
			11. Has another medical condition which in the opinion of the Investigator would preclude participation ?		
Based upon dermatologic evaluation and the information contained in Data Collection 1 and 2, the subject is:					
<input type="checkbox"/> Qualified <input type="checkbox"/> Not Qualified for participation in this study.					
Reasons for disqualification: _____					
Interviewer's Signature			Date: ____ / ____ / ____ mm dd yy		
Investigator's Signature::			Date: ____ / ____ / ____ mm dd yy		

Source Document 2
TREATMENT PHASE

Study #	Hill Top Research, Inc.	Permanent #'s
01-108591-11 CRB-01-05-065-HB		

EVENT	TIME	PROCEDURE PERFORMED ACCORDING TO PROTOCOL?
Test Contamination Procedure #2	am/pm	<input type="checkbox"/> Yes / <input type="checkbox"/> No
Test Product Treatment #2	am/pm	<input type="checkbox"/> Yes / <input type="checkbox"/> No Water Temp: °F
Test Contamination Procedure #3	am/pm	<input type="checkbox"/> Yes / <input type="checkbox"/> No
Test Product Treatment #3	am/pm	<input type="checkbox"/> Yes / <input type="checkbox"/> No Water Temp: °F
Test Contamination Procedure #4	am/pm	<input type="checkbox"/> Yes / <input type="checkbox"/> No
Test Product Treatment #4	am/pm	<input type="checkbox"/> Yes / <input type="checkbox"/> No Water Temp: °F
Test Contamination Procedure #5	am/pm	<input type="checkbox"/> Yes / <input type="checkbox"/> No
Test Product Treatment #5	am/pm	<input type="checkbox"/> Yes / <input type="checkbox"/> No Water Temp: °F
Test Contamination Procedure #6	am/pm	<input type="checkbox"/> Yes / <input type="checkbox"/> No
Test Product Treatment #6	am/pm	<input type="checkbox"/> Yes / <input type="checkbox"/> No Water Temp: °F
Test Contamination Procedure #7	am/pm	<input type="checkbox"/> Yes / <input type="checkbox"/> No
Test Product Treatment #7	am/pm	<input type="checkbox"/> Yes / <input type="checkbox"/> No Water Temp: °F
Test Contamination Procedure #8	am/pm	<input type="checkbox"/> Yes / <input type="checkbox"/> No
Recorder's Signature:		Date: ____/____/____
Reviewer's Signature:		Date: ____/____/____

Source Document 2 (continued)

TREATMENT PHASE

Study #	Hill Top Research, Inc.		Permanent #'s
01-108591-11 CRB-01-05-065-IIB			

Test Product Treatment #8	am/pm	<input type="checkbox"/> Yes / <input type="checkbox"/> No	Water Temp: °F
Test Contamination Procedure #9	am/pm	<input type="checkbox"/> Yes / <input type="checkbox"/> No	
Test Product Treatment #9	am/pm	<input type="checkbox"/> Yes / <input type="checkbox"/> No	Water Temp: °F
Recorder's Signature:		Date: ____ / ____ / ____	
Reviewer's Signature:		Date: ____ / ____ / ____	

Source Document 1
TREATMENT PHASE

Study #	Hill Top Research, Inc.		Permanent #'s
01-108591-11 CRB-01-05-065-HB			

EVENT	TIME	PROCEDURE PERFORMED ACCORDING TO PROTOCOL?	
Practice Wash	am/pm	<input type="checkbox"/> Yes / <input type="checkbox"/> No	
Decontamination	am/pm	<input type="checkbox"/> Yes / <input type="checkbox"/> No	
Base Contamination Procedure	am/pm	<input type="checkbox"/> Yes / <input type="checkbox"/> No	
Base Bacterial Sampling Procedure	am/pm	<input type="checkbox"/> Yes / <input type="checkbox"/> No	
Base Decontamination	am/pm	<input type="checkbox"/> Yes / <input type="checkbox"/> No	
Test Contamination Procedure #1	am/pm	<input type="checkbox"/> Yes / <input type="checkbox"/> No	
Test Product Treatment #1	am/pm	<input type="checkbox"/> Yes / <input type="checkbox"/> No	Water Temp: °F
Test Bacterial Sampling Procedure #1 (after first treatment)	am/pm	<input type="checkbox"/> Yes / <input type="checkbox"/> No	
Test Contamination Procedure #10	am/pm	<input type="checkbox"/> Yes / <input type="checkbox"/> No	
Test Product Treatment #10	am/pm	<input type="checkbox"/> Yes / <input type="checkbox"/> No	Water Temp: °F
Test Bacterial Sampling Procedure #2 (after 10th treatment)	am/pm	<input type="checkbox"/> Yes / <input type="checkbox"/> No	
Decontamination	am/pm	<input type="checkbox"/> Yes / <input type="checkbox"/> No	Water Temp: °F

Recorder's Signature:	Date: ____/____/____
Reviewer's Signature:	Date: ____/____/____

Data Collection Form 3

FOLLOW-UP VISIT

Study #	Hill Top Research, Inc.	Visit Code	Date	Subject Initials	Subject Screen #
01-108591-11 CRB-01-05-065-HB		Follow-up	____/____/____ mm dd yy	____/____/____ F M L	Permanent #:

Date Subject Entered the Study: ____/____/____ mm dd yy	Follow-up Visit Date : ____/____/____ mm dd yy
<p>Does the subject's hands have the presence of pimples, blisters, or raised itching bumps surrounded by erythema and/or edema that may be indicative of a skin infection ?</p> <p>YES NO If yes, complete below:</p> <p>Clinical Observations: (Include date of onset and descriptions/severity locations, etc.)</p> <p>_____</p> <p>_____</p> <p>Comments: _____</p> <p>_____</p> <p>_____</p> <p>_____</p> <p>_____</p> <p>_____</p>	
<p>Has the subject had any health related issues since the treatment procedure?</p> <p>YES NO If yes, complete below:</p> <p>Comments: _____</p> <p>_____</p> <p>_____</p> <p>_____</p> <p>_____</p> <p>_____</p>	

Investigator's Signature or designee	Date ____/____/____ mm dd yy
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Data Collection Form 4

ADVERSE EVENT

Study #	Hill Top Research, Inc.		Date	Subject Initials	Subject Screen #
01-108591-11 CRB-01-05-065-HB			mm / dd / yy	F / M / L	Permanent #:

Was reaction related to treatment? ☐ Not related ☐ Possibly related ☐ Definitely related ☐ Other (explain)

Did subject take any medication during the study period? ☐ YES ☐ NO If yes, complete section below.

Date of Onset: _____ Date Reported: _____ Date Resolved: _____

Describe event: _____

Action Taken: ☐ None ☐ Continued on study ☐ Withdrawn from the study ☐ Consulted physician

☐ Medication taken (Complete below) ☐ Hospitalized ☐ Other (explain)

Additional Comments:

FOLLOW - UP ACTION TAKEN

Date	Action Taken	Comments	Initials

CONCOMITANT MEDICATION TAKEN

Medication (Oral or Systemic)	Total Daily Dose	Start Date mm / dd / yy	Stop Date mm / dd / yy	Indication (Reason for Taking)
		/ /	/ /	
		/ /	/ /	
		/ /	/ /	

Investigator's Signature:

Recorded by:

Date

mm / dd / yy

Data Collection Form 5

PHYSICIAN'S ACTION REPORTING FORM

Study #	Hill Top Research, Inc.		Date	Subject Initials	Subject Screen #
01-108591-11 CRB-01-05-065-HB			mm dd / yy	F / M / L	Permanent #:

Date(s) of office visit(s): _____

Pertinent Medical History: (e.g., causes of similar reactions, known allergies,
potential involvement of current medications or medical conditions)

Test Product Exposure:

Use Began On: _____ Date Used Ended on: _____ Date Number of Uses: _____

Clinical Observations: (Include date of onset and descriptions severity locations, etc.)

Impression: _____

Treatment: _____

Follow Up: _____

Date Resolved: _____

Is condition related to use of the test products?

☐ Probably related*

☐ Not Related*

☐ Unknown

Reasons: _____

Physician's Signature

Date

Institution: Hill Top Research, Inc.
Investigator: Gayle K. Mulberry, M.S.

HTR Study No. 01-108591-11
Sponsor No. CRB 01-05-065-HB
Page No. I-1

Study Title: "Efficacy Evaluation of Two Liquid Soap Products In a Modified Health Care Personnel Handwash Study Versus *Escherichia coli*"

CONSENT FORM

INTRODUCTION: You are being asked to take part in a research study. Before you give your consent to be a subject, it is important that you take enough time to read and understand what your participation would involve. In preparing this consent form, it has been necessary to use some technical language. Please ask questions if there is anything you do not understand.

You will be given a signed copy of this consent form and any other necessary written information prior to the start of the study.

PURPOSE: The purpose of this research study is to determine the effectiveness of two liquid soap products containing an antibacterial ingredient against bacteria found on the skin. Approximately twenty (20) people at least 18 years of age will be screened as potential subjects in this study. At least twelve (12) subjects are expected to complete the two-visit study.

TEST ARTICLES: You will be assigned 1 of the two antibacterial liquid soap products. The liquid soap products are experimental.

STUDY PROCEDURES: Prior to enrollment in the test, you will be asked to complete a brief medical history questionnaire and another form to determine your eligibility for the study. Your hands and wrists will be checked for visible cuts, scratches or rashes on them. It is possible that you may not be able to participate based on your answers to these questions or the condition of the skin on your hands and wrists.

If you are selected to participate in this study, you will be instructed to perform a practice treatment with a liquid soap product. Then, your hands will be rinsed with alcohol, rubbed for about 15 seconds and rinsed in tap water for 15 seconds followed by drying with paper towels. Afterwards, your hands will be contaminated with a watery liquid containing relatively non-harmful bacteria (*Escherichia coli*). This liquid containing the bacteria will be spread over the surfaces of the hands, and the hands will be allowed to air dry. Following air drying, the hands will be sampled. Sampling is accomplished by having you place your hands into large plastic bags to which will be added a mild soap-like solution. A laboratory technician will massage each bagged hand for one minute. The hands will be removed from the bags and the solution from each bag will be tested to determine the number of test bacteria added to the hands. Following this baseline sampling, the hands will be rinsed for 30 seconds with tap water, rinsed with 70% alcohol and water, then dried with paper towels. Then the hands will be contaminated as above and treated with the assigned test material, 1

of the 2 liquid soap products following specific directions. After the treatment with the liquid soap product, your hands will be sampled as above about 4-5 minutes after the first treatment is completed to determine the number of bacteria removed or killed by treatment. Your hands will be contaminated and treated 10 times. After the 10th treatment, sampling will be repeated. Following each sampling, your hands will be rinsed with tap water. After the final sampling your hands will then be washed with a plain soap followed by a wash with Hibiclens®, an antimicrobial soap, and rinsed with alcohol prior to leaving the lab.

After completing the treatment visit and until your follow-up visit, you will need to check the skin on your hands each day for any pimples, bumps or rashes. Within four to nine days after you have completed treatment, you will be required to return to the lab for a follow-up visit. Your hands will be checked for infection by a technician trained in observing infection.

FEMALES OF CHILDBEARING POTENTIAL: You may not participate in this study if you are pregnant or nursing. As part of giving your consent you must agree to have a urine pregnancy test at the start of the study.

RISKS: The risks associated with this test are primarily related to infection with the test bacteria. For healthy persons, the possibility of a skin infection exists; however, this possibility is remote because, (1) test bacteria are applied only to healthy or uninjured skin, and (2) the skin is cleansed with antibacterial products following contact with the test bacteria. Your hands may also show a "reaction." A "reaction" could be pimples, blisters or raised bumps surrounded by redness and/or swelling. It is unlikely, but possible, that a rash could develop.

No risks to you as a study participant, other than those described above as "reactions," are anticipated during the study. Reactions are usually due to irritation, although an allergic reaction might occur. If you become allergic, it is possible that future exposures to the same ingredient may cause a skin reaction. If this occurs, you will be provided with information to minimize the chance for future exposures.

You may experience risks or side effects that are not known at this time. You will be informed in a timely manner if new information becomes available that may influence your willingness to continue in this study.

BENEFITS: You will not benefit from the application of test product but the study results may allow a new or improved product to be marketed.

ALTERNATIVE PROCEDURES/TREATMENTS: Because you are not being treated for a medical condition, alternative treatments do not apply to this study.

CONFIDENTIALITY: Information concerning you that is obtained in connection with this study will be kept confidential by Hill Top Research, except that the sponsoring company whose product is being tested will receive a copy of the study records. The records will be coded to protect your identity. In addition, government regulatory agencies, including the U.S. Food and Drug Administration (FDA), may inspect the records of the study. Information obtained in the study may be used for medical or scientific publication, but your identity will remain confidential.

MEDICAL TREATMENT: If in the course of this study you experience illness, discomfort or injury that appears to be a result of the study, Hill Top Research will provide you with medical care at no cost to you. Providing such medical care is not an admission of legal responsibility. If such illness, discomfort or injury does occur, ask any staff member to arrange a meeting for you with the appropriate personnel.

In certain cases of illness or injury resulting from this study, workers' compensation coverage may be available. In accordance with Ohio law, Hill Top Research has secured workers' compensation coverage for participants in its studies and tests, and has paid and will pay appropriate premiums into the State Insurance Fund on behalf of such participants.

WHO TO CONTACT: If you have any questions about this study or in case of an emergency, contact Emilie, Study Coordinator at 513-831-3114, ext. 2324 during business hours (M-F, 8:00 A.M. - 5:00 P.M.) or Ann Brady, Study Manager at 513-831-3354 after hours.

VOLUNTARY PARTICIPATION/WITHDRAWAL: Your participation in this research study is strictly voluntary. You may refuse to participate or may discontinue participation at any time during the study without penalty or loss of benefits to which you are otherwise entitled.

If you agree to participate in this study, you are also agreeing to provide Hill Top Research with accurate information and to follow study instructions as given to you. If you fail to comply with study procedures, your participation may be terminated.

Your participation in the study may be discontinued at any time without your consent by the Investigator, the FDA, or the sponsoring company.

COMPENSATION: You will be paid \$55.00 for the completion of this study. You will be compensated according to the following schedule:

If you do not qualify	Visit 1	you will receive	\$10.00
If you qualify but are eliminated as an extra subject	Visit 1	you will receive	\$15.00
If you complete	Visit 1	you will receive	\$30.00
If you complete	Visit 2	you will receive	\$55.00

Payments will be made at the end of the study.

There are no anticipated expenses to you for participating in this study. All test related materials will be provided at no cost to you.

CONSENT TO PARTICIPATE

I know that my participation in this study is voluntary and that I have the right to refuse to participate. I know that I may withdraw from the study at any time without penalty or loss of benefits to which I am otherwise entitled. If I withdraw or am dismissed for failure to obey rules or follow directions, I understand I will only be paid for the portion of the study that I have completed. If, in the judgment of the Investigator, it is best to discontinue my participation in the study for other reasons, I will be paid either in full or for that portion of the study already completed.

If I am a female of childbearing potential, I am not currently pregnant or nursing an infant. I am using an adequate means of birth control and, if I become pregnant or believe I have become pregnant, I will notify the Investigator immediately.

CONSENT: I have read all of the above information and have been given an opportunity to ask questions about this study. Answers to such questions (if any) were satisfactory. I am eighteen years of age or older and freely and without reservation give my consent to serve as a subject in this study. By signing this form, I have not given up any of my legal rights as a research subject.

Subject's Name Printed: First Middle Initial Last

Subject's Signature Date

Signature of Person Conducting Consent Discussion Date

SUBJECT SCREEN NO. _____

SUBJECT NO. _____

PILOT STUDY

NON-RINSE OFF FORMULATION

SWH094-136, SWH094-137

Clinical Study Report

CRA: Kathy Wiandt

Date: May 22, 2001

Study Statistician: Jeanne Philippo

Retention Limit: Until Superseded

Approved by: JB 5/30/01

Subject: Results of Efficacy Evaluation of Three Handwipe Products in a Modified Healthcare Personnel Handwash Study Versus *Escherichia coli* – CRB-01-04-063-HB / HT# 01-108589-11.

Objective:

The objective of this study was to determine the ability of three antibacterial handwipe products to significantly reduce transient microbial flora (*Escherichia coli* 11229) on the hands after a single treatment and after ten (10) treatments.

Materials Tested:

Test Code	Test Material	Active Ingredient	Batch Number
A	Handwipe Product	1% Salicylic Acid	SWH94-136
B	Handwipe Product	0% Salicylic Acid	SWH94-137
C	Handwipe Product	2% Salicylic Acid	SWH94-138

Key Conclusions:

The purpose of this test was to screen antibacterial handwipe prototypes. The base size per product tested was small and therefore no statistical differences were determined between the products.

The summary of the mean logs recovered and the log reductions achieved following the first and tenth washes were determined.

Table I – Summary of HCPHWT Log ₁₀ Bacterial Results								
		Baseline	Log ₁₀ Counts – 1 Wash			Log ₁₀ Counts – 10 Washes		
Treatment	Sample Size	Mean	Mean	Change from Baseline	% Reduction	Mean	Change from Baseline	% Reduction
A-Handwipe 1% Salicylic Acid	4	7.43	5.54	1.89	98.7	4.05	3.38	>99.9
B-Handwipe 0% Salicylic Acid	4	6.78	5.35	1.43	96.3	4.34	2.44	99.6
C-Handwipe 2% Salicylic Acid	4	7.45	5.43	2.01	99.0	4.44	3.01	99.9

Attached are the statistical analysis tables for the study.

Study Summary:

Test Site: Hill Top Research, Miamiville, Ohio

Study Dates: April 20-24, 2001

Investigator: Gayle K. Mulberry, M.S.

Experimental Design: This was a randomized clinical study consisting of a one day test period and a single follow-up visit. Three test products were evaluated. Four subjects were used to evaluate each product.

Efficacy Measurements Taken: The subjects' hands were contaminated with a suspension of *E.coli*. Subjects' hands were contaminated eleven (11) times and sampled three times using a plastic bag sampling procedure. The first contamination and sampling was for the determination of the base count. The second contamination and sampling was for determination of the test count after one treatment with the assigned Test Product. After eleven contamination steps and ten treatments with the assigned Test Products the hands were sampled using the plastic bag sampling procedure.

Subject Demographics: Twelve (12) male and female subjects, ≥ 18 years old, who do not regularly use antibacterial/antimicrobial soaps, medicated lotions or creams and or antidandruff shampoos were enrolled into the study. Four (4) subjects were used to evaluate one of three test products.

Overview: To become familiar with the wipe procedure using non-medicated hand wipe, the subjects practiced the wipe procedure with Nice 'n' Clean®. For the base count, subjects' hands were contaminated with *E. coli*. Immediately following the contamination step, the organisms on the subjects' hands were removed using a plastic bag sampling procedure.

Prior to each treatment wash, subjects' hands were contaminated with *E. coli*. After completing the contamination step, the subjects performed the test product application procedure with the assigned Test Product. The subjects wiped each of their hands for fifteen (15) seconds. Approximately five (5) minutes following the wipe procedure, the organisms on both of the subjects' hands were removed using a plastic bag sampling procedure. Approximately five (5) minutes following the tenth treatment, the organisms on the subjects' hands were removed using a plastic bag sampling procedure.

Samples of the subjects' sampling solutions were diluted, plated, and incubated. Following incubation, the numbers of colony forming units (CFU's) were enumerated. Antibacterial activity was determined by comparing the number of bacteria removed from the hands after one (1) treatment with the assigned Test Product and ten (10) treatments with the assigned Test Product to the number of bacteria removed from unwashed hands.

Data Analysis:

For the bag juice results, each subject's base sampling CFU's was compared to their test sampling CFU's using a nonparametric Wilcoxon paired signed-rank test. P-values ≤ 0.10 were considered statistically significant. Percent change for each organism was computed by the following formula:

$$1 - \left(\frac{\text{geometric mean of the test CFU's}}{\text{geometric mean of the baseline CFU's}} \right) \times 100$$

Regulatory/Ethics Status:

This study was conducted in compliance with federal, state, and local regulations, guidelines, and standards including those related to Informed Consent and Good Clinical Practices as specified under 21 CFR 321.66.

Subject Accountability:

Eighteen (18) subjects were screened for the study. Twelve (12) subjects were screened, enrolled and completed this study. Four (4) subjects met the study qualifications, but were excluded because they were extra subjects. Two (2) subjects were excluded because of open cuts on their hands.

Adverse Events:

There were no adverse events in this study.

K. W. Traudt

Clinical Research Associate

Jeanne C. Philippo

Statistician

CRB-01-04-063-HB

			Baseline Log10(Count)			Final Log10(Count)		
Wash	Treatment	Sample Size	Mean	Median	Std.Error	Mean	Median	Std.Error
1	A:	4	7.43	7.41	0.053	5.54	5.54	0.140
	B:	4	6.78	6.68	0.229	5.35	5.44	0.284
	C:	4	7.45	7.46	0.048	5.43	5.40	0.069
10	A:	4	7.43	7.41	0.053	4.05	4.08	0.341
	B:	4	6.78	6.68	0.229	4.34	4.31	0.227
	C:	4	7.45	7.46	0.048	4.44	4.98	0.660

			Change in Log10(Count)				
Wash	Treatment	Sample Size	Mean	Median	Std.Error	P-Value	Percent Reduction
1	A:	4	1.89	1.91	0.133	0.1250	98.7
	B:	4	1.43	1.46	0.125	0.1250	96.3
	C:	4	2.01	2.11	0.108	0.1250	99.0
10	A:	4	3.38	3.37	0.344	0.1250	>99.9
	B:	4	2.44	2.46	0.055	0.1250	99.6
	C:	4	3.01	2.41	0.662	0.1250	99.9

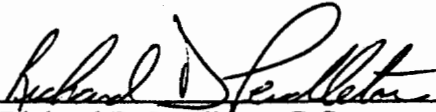
Summary of HCPHWT Log10 Bacterial Results								
			Log10 Counts - 1 Wash			Log10 Counts - 10 Washes		
Treatment	Sample Size	Baseline Mean	Mean	Change from Baseline	Percent Reduction	Mean	Change from Baseline	Percent Reduction
A:	4	7.43	5.54	1.89	98.7	4.05	3.38	>99.9
B:	4	6.78	5.35	1.43	96.3	4.34	2.44	99.6
C:	4	7.45	5.43	2.01	99.0	4.44	3.01	99.9

HTR Study No.: 01-108589-11
Sponsor Study No.: CRB-01-04-063-HB

QUALITY ASSURANCE STATEMENT

This study was inspected in accordance with the Standard Operating Procedures of Hill Top Research, Inc. To assure compliance with the study protocol, the Quality Assurance Unit performed an inspection during the conduct of this study and completed an audit of the study records.

Data reviewed by:


Richard D. Pendleton, B.S.
Auditor, Quality Assurance

5/2/01
Date

CLINICAL STUDY PROTOCOL

Clinical Research & Biometrics Department
Sharon Woods Technical Center
Cincinnati, Ohio 45241

Title: Efficacy Evaluation Of Three Handwipe Products In A Modified Health Care Personnel Handwash Study Versus *Escherichia Coli*

Study Number: CRB-01-04-063-HB / HT# 01-108589-11

Issue Date: 4/18/01

Products Tested: Antibacterial Handwipe Prototype
Antibacterial Handwipe Prototype
Antibacterial Handwipe Prototype

Test Facility: Hill Top Research, Inc.
Main and Mill Streets
Miamiville, Ohio 45147

Microbiology Samples The Procter and Gamble Company
Miami Valley Laboratories

Principal Investigator: Gayle Mulberry, M.S.

Sub-Investigators: Kathleen A. Baxter, B.S.
Ann R. Brady, A.S.

Test Sponsor: The Procter & Gamble Co., Inc.
Sharon Woods Technical Center
11520 Reed Hartman Highway
Cincinnati, Ohio 45241

		<u>work</u>	<u>home</u>
Sponsor Toxicologist:	Candace Doepker, Ph.D.	(513) 626-5536	
Sponsor Representative/CRA:	Kathy Wiandt, B.S.	(513) 626-5225	(513) 398-6035
Sponsor Statistician:	Jeanne Philipppo, B.A.	(513) 626-5937	
Expected Study Start Date:	April 20, 2001		
Expected Study End Date:	April 24, 2001		

I. Study Objective and Background

A. Objective

The objective of the study is to determine the ability of a three antibacterial handwipe products to significantly reduce transient microbial flora (*Escherichia coli* 11229) on the hands after a single treatment and after ten (10) treatments.

B. Background

The skin microflora can be divided into two (2) groups, the resident flora and the transient flora. The resident flora includes organisms that are consistently present on the skin. The transient flora are the contaminating skin organisms resulting from contact with the environment. They comprise a wide variety of Gram positive and Gram negative species that can be responsible for the spread of infections and gastrointestinal diseases.

Since the benefits that result from washing with antibacterial products can not be easily measured under consumer use conditions, it is necessary to do controlled clinical studies to demonstrate their efficacy. This clinical study is a modification of an ASTM test method, "Evaluation of Health Care Personnel Handwash Formulation"⁽¹⁾ and reported in the Tentative Final Monograph for Health Care Antiseptic Drug Products⁽²⁾. It is used to determine the ability of an antimicrobial handwashing agent, when used in a hand washing procedure, to reduce the transient microbial flora (contaminants). This study is designed to demonstrate the efficacy of three towelette products in reducing the numbers of a marker organism, *Escherichia coli* ATCC 11229 on the hands after a contamination and a single treatment and after ten treatments. Efficacy is determined by comparing the numbers of marker organisms on the hands before and after using the test products.

C. Study Safety Statement

This testing meets the ethical requirements stipulated in the Sponsor's Policy for Research Involving Human Subjects. Appropriate safety testing has been completed and risk assessments justify the placement of the test products in this study at these concentrations (levels of exposure).

II. Study Summary

A. Overview

This randomized clinical study will consist of a one day test period and a follow-up visit. Three (3) test products will be evaluated. Twelve (12) male and female subjects, ≥ 18 years old, who do not regularly use antibacterial/antimicrobial soaps, medicated lotions and creams, and or antidandruff shampoos (Appendix D), will be enrolled into the study. Four (4) subjects will be used to evaluate each test product.

On the day of the study, the subjects will report to the clinical test facility. During this period, subjects' hands will be contaminated with a suspension of *E. coli*. Subjects' hands will be contaminated eleven (11) times and sampled three (3) times using a plastic bag sampling procedure. The first contamination and sampling will be for the determination of the base count. The second contamination and sampling will be for determination of the test count after one (1) treatment with the assigned Test Product. After eleven (11) contamination steps and ten (10) treatments with the assigned Test Products the hands will be sampled using the plastic bag sampling procedure.

To become familiar with the wipe procedure using the towelette product, subjects will begin the test procedure by first performing a practice wipe with Nice 'n' Clean®. For the base count, subjects will have their hands contaminated with *E. coli*. Immediately following the contamination step, the organisms on the subjects' hands will be removed using a plastic bag sampling procedure.

Prior to each treatment wash, subjects' hands will be contaminated with *E. coli*. After completing the contamination step, the subjects will perform the test product application procedure with the assigned

Test Product. Approximately five (5) minutes following the first procedure, the organisms on both of the subjects' hands will be removed using a plastic bag sampling procedure. Approximately five (5) minutes following the tenth treatment, the organisms on the subjects' hands will be removed using a plastic bag sampling procedure.

Aliquots of the subjects' sampling solutions will be diluted, plated, and incubated. Following incubation, the number of colony forming units (CFU's) will be enumerated. Antibacterial activity is determined by comparing the number of bacteria removed from the hands after one (1) treatment with the assigned Test Product and ten (10) treatments with the assigned Test Product to the number of bacteria removed from unwashed hands.

B. Study Schedule

1. Subject Qualification and Enrollment

Prospective subjects will visit the test facility to be screened for their eligibility to participate in the study. Eligibility will be based upon information provided in the Demographics/Dermatological/Medical History Form (DCF 1) and the Inclusion/Exclusion Form (DCF 2); and completion of a written informed consent (Appendix A).

2. Test Period

Subjects continuing on the study will be assigned a permanent subject number. Subjects will be assigned to one of the three test products according to the study randomization.

The following outlines the schedule of procedures for the test day:

1. Subjects will perform a practice wipe with Nice 'n' Clean ®.
2. Subjects will rinse their hands with 70% alcohol and rinse their hands under running tap water (Section G).
3. Subjects' hands will be contaminated (Section E).
4. Subjects' hands will be sampled for a base count (Section F).
5. Subjects will rinse their hands with water for 30 seconds (Section G).
6. Subjects will rinse their hands with 70% alcohol and rinse with tap water (Section G).
7. Subjects' hands will be contaminated (Section E).
8. Subjects will wipe their hands following the wipe procedure for the assigned Test Product (Section C).
9. Subjects' hands will be sampled for a treatment value four (4) minutes and thirty (30) seconds \pm thirty (30) seconds after the first wipe with the assigned Test Product (Section F).
10. The hands will be rinsed for thirty seconds.
11. Subjects will perform steps 7 and 8 (above) a total of nine (9) more times at a minimum of five (5) minutes between each wash procedure.
12. The subjects' hands will be sampled for a treatment value four (4) minutes and thirty (30) seconds \pm thirty (30) seconds after the tenth wipe with the assigned Test Product (Section F).
13. Subjects' hands will be disinfected with soap and water wash and Hibiclens® (4% chlorhexidine gluconate) wash and with a 70% alcohol rinse (Section G).

Note: A detailed schedule of the above procedures can be found in Appendix C.

To ensure that any delayed adverse events, such as primary skin infections, are reported to the Study Investigator, all test subjects will be given a copy of Subjects' Instructions Following Study Completion (Appendix B) before leaving the clinical site after they have completed the study. This

sheet will instruct the subjects to examine their hands and wrists daily until the final scheduled visit for the presence of pimples, blisters, or raised, red itching bumps surrounded by erythema and/or edema that may be indicative of a skin infection. Subjects, who notice such lesions, will be instructed to call the clinical test site. The subjects will return to the clinical test site within four (4) to nine (9) days after the study procedures have been completed to have their hands and wrists examined by a technician. The technician will complete DCF 3 for each subject on their follow-up visit.

C. Product Treatment Procedure

Subjects will wipe their hands and wrists according to the procedure below. A record of the product treatment procedure will be documented on Source Documents 1 or 2

1. The technician will dispense the Test Product into the subject's left hand using a gloved hand.
2. The subject will rub all surfaces of their right hand and wrist for fifteen (15) seconds while the technician instructs the subject to:
 - rub palm
 - rub back of hand
 - rub the wrist
 - rub fingers and web areas between fingers
 - rub the tips of the fingers
3. The subject will transfer the wipe to their right hand.
4. The subject will rub all surfaces of their left hand and wrist for fifteen (15) seconds while the technician instructs the subject to:
 - rub palm
 - rub back of hand
 - rub the wrist
 - rub fingers and web areas between fingers
 - rub the tips of the fingers

D. Preparation of Bacterial Suspensions

A stock culture of *Escherichia coli*, ATCC 11229, will be prepared by transferring one (1) colony from an agar plate or slant aseptically to a tube containing sterile Trypticase Soy Broth (TSB). The inoculated broth will then be incubated for 24 ± 4 hours at $35 \pm 2^\circ$ C. At least three (3) additional 24 hour broth transfers will be made in tubes containing appropriate volumes TSB from this broth culture.

A 2-liter flask containing 1000 mL of TSB will be inoculated with 1.0 mL of the final 24 hour broth transfer. The flask will be incubated for 24 ± 4 hours at $35 \pm 2^\circ$ C. Prior to any withdrawal of culture, whether for hand contamination or for numbers assay, the suspension will be stirred or shaken. The suspension will be assayed for number of organisms at the beginning and end of the treatment period. A suspension will not be used for more than eight (8) hours.

E. Contamination

Note: Prior to contamination, subjects hands must be visibly dry. Also, care should be taken to ensure that the culture is evenly spread over both hands

A total volume of 4.5 mL of the assigned bacterial suspension will be dispensed into the subjects' cupped hands in 1.5 mL increments. After each 1.5 mL aliquot is added, the suspension will be rubbed thoroughly over the surface of both hands, not going above the wrist and avoiding the nail beds. Each application and spreading should last approximately twenty (20) seconds. Between each aliquot the hands will be held away from the body and allowed to air dry for approximately thirty (30) seconds.

Following the third 1.5 mL aliquot, the hands are allowed to air dry for approximately one (1) minute. A record of base and test contaminations will be documented on Source Document 1 or 2.

F. Bacterial Sampling Procedure

For removal of bacteria from the subjects' hands, loose fitting plastic bags with low bioburden will be placed on each subject's right and/or left hands. A 75 mL aliquot of stripping solution [0.1% Triton X-100 in 0.075 M phosphate buffer, 0.5% polysorbate (Tween) 80, 0.07% Lecithin, pH 7.9] will be aseptically added into each bag. The same solution will be used for the base counts and test counts.

The bag on each hand will be secured at the wrist with a child's size tourniquet and massaged for one (1) minute in a uniform manner by a lab technician. Aliquots of the solution will be aseptically obtained directly from the bag without touching the hands in the process and will be appropriately diluted in a sterile diluent with the appropriate neutralizer (for the test wash samples only). A record of base and test samplings will be documented on Source Document 1.

The solution samples for bacteria counts will be labeled by either an Investigator derived code or the actual subject's number so that the individuals who prepare the plates and count the CFU's are unaware of the sources of the sampling solution.

The solution will be aseptically placed in a sterile test tube. The test tube will be affixed with the subject number, baseline or post-treatment, and placed on ice for microbiological analysis. The sponsor will analyze the samples for microbiological content. The transfer of the microbial specimens will be recorded on Source Document 3.

G. Disinfection of Hands

After the baseline sampling, the subjects will rinse their hands for thirty (30) seconds under running tap water. The subjects' hands will be disinfected with a 70% alcohol wash. Subjects' hands will be squirted with 70% alcohol for approximately ten (10) seconds. Subjects will rub the alcohol over the surface of their hands and wrists for approximately fifteen (15) seconds. Subjects will rinse their hands and wrists under running tap water for approximately fifteen (15) seconds and dry their hands and wrists with paper towels.

After the final sampling is completed, the subject's hands will be washed with a bland soap (provided by the investigator) for approximately for thirty (30) seconds and rinsed for approximately fifteen (15) seconds. The subjects' hand will then be washed with Hibiclens® (4% chlorhexidine gluconate) for at least sixty (60) seconds. Subjects' hands and wrists will be rinsed with a 70% alcohol wash for ten (10) seconds. The subjects will rub the alcohol on all surfaces of their hands for fifteen (15) seconds and allow their hands to air dry.

A record of each disinfection procedure will be recorded on Source Document 1.

H. Plating and Incubation of the Organisms

Baseline specimens will be serially diluted in half-strength (0.0375 M) buffer (without Triton X-100) in ten-fold dilutions to 10^{-3} , 10^{-4} and 10^{-5} . The diluted specimens will be plated using an automated plating system (Eddyjet system) onto MacConkey's agar. Post treatment specimens will be serially diluted in ten-fold dilutions to 10^{-1} , 10^{-2} , 10^{-3} and 10^{-4} . Using an automated plating system (Eddyjet system), the undiluted and diluted specimens will be plated onto MacConkey's agar. The media for these analyses are shown in Appendix E.

Plated samples will be incubated aerobically for 18 - 24 hours at $35 \pm 2^{\circ}\text{C}$. The plates will be analyzed using the Counterstat®. The results will be reported as colonies per mL using the Counterstat® software package.

The results will be recorded in an electronic file created by the sponsor.

III. Study Population

Subjects will be screened for their eligibility to participate based upon information provided in the Demographics/Dermatological/Medical History Form [Data collection form (DCF) 1]. Only subjects meeting the inclusion/exclusion criteria, outlined in DCF 2, will be allowed to participate in the study. If a subject is admitted to this study in apparent violation of any of the above criteria, the reason(s) for admission will be noted by the Investigator or her designee.

A. Subject Inclusion Criteria

Subjects will be eligible for enrollment if they:

1. Are a male or female, over 18 years of age ;
2. Have signed a written informed consent (Appendix A);
3. Are in good health, as evidenced by response to the Demographics/Dermatological/ Medical History Form (DCF 1);
4. Have hands and wrists that are free of dermatoses, cuts, lesions, and other skin disorders;
5. Are willing to comply with all study protocol requirements.

B. Subject Exclusion Criteria

Subjects will not be enrolled in the study if they:

1. Are currently participating in another clinical study at this or any other facility;
2. Have participated in any type of arm or hand wash study within the past 7 days;
3. Have cuts, lesions, or other skin disorders on their hands or wrists;
4. Have soap, detergent, antibiotic, and/or perfume allergies;
5. Have eczema or psoriasis on their hands or arms;
6. Are using antibacterial/antimicrobial soaps (liquids and/or bars), medicated lotions and creams, and/or anti-dandruff shampoos in the home within the last week (Appendix D);
7. Have excessively long or artificial nails (≥ 2 mm free edge) which would interfere with sampling;
8. Are currently pregnant;
9. Are currently lactating;
10. Have been diagnosed as having a medical condition which would preclude participation such as: diabetes, hepatitis, an organ transplant, or AIDS (or HIV positive); and/or
11. Have any other medical condition, which in the opinion of the Investigator, would preclude participation.

D. Subject Number Assignment and Randomization

Upon entry into the study, each subject will be assigned a screening number beginning with 1001. Subjects will be assigned a permanent consecutive number, beginning with 001, as they are accepted into the study. This number will be used to identify the subject for the duration of the study.

IV. Study Material

A. Test Product

The test products will be sent by the Sponsor to the clinical site prior to study initiation. The test products will be identified with the appropriate label affixed to the outside of each container.

B. Shipping of Treatment Products and Other Study Supplies

The quantity of all treatment products and other study supplies, shipped to and returned from the clinical site, will be documented by the test site. The treatment products will be packed into one or more cartons labeled with:

1. the study number;
2. distributor statement (i.e., "Distributed by Hill Top Research, Inc." with the facility's full address and phone number);
3. any applicable safety and handling procedures.

C. Return of Study Materials

Upon completion of the study, the Investigator(s) will insure that all test products and study materials, whether completely used, partially used, or unused will be returned to the Sponsor at the following address:

The Procter & Gamble Company
Sharon Woods Technical Center
11520 Reed Hartman Highway
Cincinnati, Ohio 45241
Attn.: Kathy Wiandt

V. Other Study Documentation

A. Adverse Events

Should any unexpected or serious adverse event occur during the clinical study or as a result of test product or study procedures, the subject will be requested to return to the site to be examined by the investigator or designee. The Investigator will determine if the adverse event is likely to be associated with product treatment or the study procedures. The investigator or other qualified medical personnel will determine if the event warrants termination of participation and/or to prescribe treatment, if necessary. The Investigator will notify the Sponsor representatives, Ward L. Billhimer, 513-626-1926 (work) or 513-831-8163 (home) or Kathy Wiandt, 513-626-5225 (work) or 513-398-6035 (home).

Each subject will need to be followed until the resolution of any adverse event. Information pertaining to the presenting signs, working diagnosis, assessment of the relationship of the adverse event to the product treatment, results of the follow-up visits and any prescribed treatment, will be documented in DCF 4. If treatment by a physician is necessary, this treatment will be documented on DCF 5.

The following criteria will be used to determine the reporting time frame.

1. Any serious adverse events or adverse events requiring immediate medical attention will be reported to the Sponsor's Monitor immediately (night or day) by telephone.
2. Adverse events resulting in subject termination from the study will be reported during the immediate business day by telephone.
3. Adverse events that do not require discontinuation of test participation can be reported during the immediate business day or next business day by telephone.

4. In the event of a serious adverse reaction, not necessarily related to use of the test product, or in the event of a death from any cause, the Investigator must report the event to the Sponsor's Monitor.

B. Protocol Amendments

If it becomes necessary to modify this protocol, the modification will be documented by a protocol amendment signed by the investigator and a representative of the Sponsor. All amendments to the final protocol will be consecutively numbered and will describe any changes made and the rationale for making the changes.

C. Protocol Deviations

If a deviation from the final protocol occurs, it is the responsibility of the Investigator, or designee, to notify the Clinical Research Associate or designee. The deviation and subsequent notification will be documented appropriately.

D. Study Monitoring

The Investigator will permit a representative of the Sponsor (usually the Clinical Research Associate) to visit the facility during the course of the study to monitor study progress. During the visit(s), the Investigator will permit the monitor to inspect all forms and corresponding study subject's records to verify adherence to the protocol. The study monitor will also be permitted to review and verify test articles, wash procedure, and any Investigator-generated or Sponsor-generated study documents. The monitor will document and discuss this visit with the Investigator, or his designee, including any problems that are to be resolved.

VI. Statistical Analyses

The sponsor will be responsible for all statistical analyses. For the bag juice results, each subject's base sampling CFU's will be compared to their test sampling CFU's using a nonparametric Wilcoxon paired signed-rank test. P-values ≤ 0.10 will be considered statistically significant. Percent change for each organism will be computed, if needed, by the following formula:

$$\left(\frac{1 - \text{geometric mean of the test CFU's}}{\text{geometric mean of the baseline CFU's}} \right) \times 100$$

VII. Investigator Responsibilities

A. Subject Informed Consent

All subjects will be informed as to the type of study, the general nature of the products being tested, and any known or anticipated adverse reactions that might result from participation. Each subject must provide the Investigator with written informed consent to serve as a participant in the study. Basic elements of informed consent are outlined in 21 CFR 31.25.

B. Final Report

The Sponsor will generate a final report of clinical results. The investigator will provide a detailed description of the adverse events and deviations from the protocol. The investigator will also include an accounting of the subjects screened, eliminated, enrolled and terminated. The Investigator will submit the legible copies of all data collection forms. The Sponsor may request one (1) copy of all case report forms before the Investigator's report is ready for submission to the Sponsor.

C. Record Retention

The Investigator will retain all study records in accordance with the test facility's SOP's.

D. Confidentiality

The Investigator and employees of the test facility are obligated to keep any information confidential regarding any of the personal cleansing products and all aspects of the study, as subject to the terms and conditions of the Laboratory Services Agreement between the test facility and Sponsor.

VIII. References

1. *Annual Book of ASTM Standards*, Volume 11.04, ASTM Designation: E 1174-94, Standard Test Method for "Evaluation of Health Care Personnel Handwash Formulation".
2. Tentative Final Monograph for Health-Care Antiseptic-Drug Products; Proposed-Rule, 21-CFR Parts 333 and 369, *Federal Register*, Volume 59, No. 116, June 17, 1994.

IX. Attachments

The following Appendices, Data collection forms are included as attachments to the Final Protocol:

- A Written Informed Consent
- B Subject's Follow-up Instructions
- C Schedule of Test Period Procedures
- D List of Representative Antibacterial/Antimicrobial Products
- E Microbiological Media and Methods

Data Collection Forms

- 1 Demographics/Dermatological Medical History Form
- 2 Inclusion/Exclusion Form
- 3 Follow- up Visit
- 4 Adverse Event
- 5 Physician's Report Form

Source Documents

- 1 Treatment Phase (Baseline, Wash 1 and Wash 10)
- 2 Treatment Phase (Washes 2 through 9)
- 3 Shipping of Microbiological Specimens

X. Sponsor and Investigator Concurrence

For The Procter and Gamble Company

PREPARED BY:

Kathy Wiandt
Kathy Wiandt, B.A., Clinical Research Associate
Clinical Research and Biometrics Department

Date: 4/18/01

STATISTICIAN:

Jeanne C. Philippo
Jeanne C. Philippo, B.A., Statistician
Clinical Research and Biometrics Department

Date: 4/18/01

APPROVED BY:

Ward L. Billhimer
Ward L. Billhimer, M.S., Senior Scientist
Clinical Research and Biometrics Department

Date: 4/18/01

Agreed and Accepted by Hill Top Research, Inc. and the Study Investigator(s) for
CRB-01-04-063-HB:


I certify that I have reviewed and approved the protocol, informed consent form, and other associated documents and agree to abide by their terms. In addition, I agree to conduct this clinical study in compliance with federal, state and local government regulations, guidelines and standards applicable to such studies.


Gayle K. Mulberry, M.S., Investigator

Date: 4/19/01


Kathleen A. Baxter, B.S., Sub-Investigator

Date: 4.19.01


Ann R. Brady, A.S., Sub-Investigator

Date: 4.18.01

Appendix A
HT-01-108589-11
CRB-01-04-063-HB

WRITTEN INFORMED CONSENT

To be provided by the clinical site.

Appendix B
HT-01-108589-11
CRB-01-04-063-HB

SUBJECT'S INSTRUCTIONS FOLLOWING STUDY COMPLETION

You have just completed participation in a clinical study, "Efficacy Evaluation Of Three Handwipe Products Modified Health Care Personnel Handwash Study Versus *Escherichia Coli*". During this study, a quantity of bacteria (*E. coli*) was placed on the surface of both your hands. Although we do not expect you to have any adverse experience as a result of participation in this study, there is a remote possibility that an infection may develop on your hands and wrists within four (4) to nine (9) days.

To determine whether you have developed an infection from the test bacteria, we would like you to examine your hands and wrists daily. If you notice the appearance of any pimples, blisters or raised bumps surrounded by redness and/or swelling, please contact Gayle Mulberry or Ann Brady at (513) 831-3114 during normal business hours (8:00 am-5 p.m.) or at (513) 831-3354 after hours.

You are required to return to the test site for a follow-up visit. Your follow-up is scheduled for:

Date

Time

Thank you for your cooperation.

Appendix C
HT-01-108589-11
CRB-01-04-063-HB

SCHEDULE OF TEST PERIOD PROCEDURES

- 1. Practice treatment Nice 'n' Clean®:**
 - towelette is placed in subjects' left hand
 - subject will rub all surfaces of their right hands and wrist for 15 seconds including palmar surface, back of hand, fingers and web area between fingers, and finger tips
 - subject transfers towelette to right hand
 - subject will rub all surfaces of their left hands and wrist for 15 seconds including palmar surface, back of hand, fingers and web area between fingers, and finger tips
- 2. 70% alcohol rinse**
 - squirt backs and palms of subjects' hands with 70% alcohol for 10 seconds
 - subjects rub alcohol over hands for 15 seconds
 - subjects rinse hands under running tap water for 15 seconds
 - subjects dry hands with paper towels
- 3. Base contamination**
 - dispense 1.5 mL aliquot of bacterial suspension onto both subjects' hands
 - subjects rub aliquot over hands for 20 seconds
 - allow subjects' hands to air dry for approximately 30 seconds
 - repeat application 2 times
 - allow subjects' hands to air dry 1 minute after the last application
- 4. Base sampling**
 - place bags on subject's right and left hands
 - dispense 75 mL stripping solution into each bag
 - secure bags
 - massage for 1 minute
 - sample each bag
- 5. Water rinse**
 - subjects rinse hands with water for 30 seconds
- 6. 70% alcohol rinse**
 - perform as above
- 7. Test contamination (prior to Test Product treatments 1 through 10)**
 - perform as above under base contamination
- 8. Test Products Treatments (treatments 1 through 10)**
 - perform as described under practice treatment
 - for treatments #1 and #10, hands will not be dried prior to sampling
 - for treatments # 2 through #9 subjects will dry hands with paper towels
- 9. Test sampling - Following Treatment 1**
 - perform as above under base sampling

- subjects rinse hands with water for 30 seconds after the first test sampling

Appendix C (continued)

HT-01-108589-11

CRB-01-04-063-HB

10. Test sampling - Following Treatment 10

- place bag on of the subject's hands
- dispense 75 mL stripping solution into the bag
- secure bag
- massage for 1 minute
- sample bag

11. Disinfection

- subject rinse hands for thirty (30) seconds
- squirt subjects' hands with 2 mL of bland soap
- subjects wash hands and wrists for approximately 30 seconds
- subjects rinse hands and wrists for approximately 15 seconds
- squirt subjects' hands with 5 mL of Hibiclens[®]
- subjects wash hands and wrists for at least 60 seconds
- subjects rinse hands and wrists for 15 seconds
- squirt backs, palms and wrists of subjects' hands with 70% alcohol for 10 seconds
- subjects rub alcohol over hands and wrists for 15 seconds
- subjects' hands will be allowed to air dry

Appendix D
HT-01-108589-11
CRB-01-04-063-HB

LIST OF ANTIBACTERIAL / ANTIMICROBIAL PRODUCTS

Medicated Acne Cleansers

Benzac W Wash 5
Desuam-X 5 Wash
Benzac W Wash 10
Desquam-X 10m Wash
Fostex 10% BPO Wash
Oxy 10 Wash
Propa P.H. Liquid Acne Soap
PanOxyl 5
Fostex 10% BPO
PanOxyl 10
Clearasil Antibacterial Soap
Sastid Plain Therapeutic Shampoo and Acne Wash
Oxy Clean Soap
Fostex Medicated Cleansing Bar
Salicylic Acid and Sulfur Soap
Sulfur Soap

Antidandruff Shampoos

Head and Shoulders (all formulas)
Selsun Blue (all formulas)
Pert Plus for Dandruff
Suave for dandruff
Neutrogena T-gel
Neutrogena T-sal
Scalpacin
Tegrin
Any antidandruff shampoo

Anti-bacterial Soaps

Safeguard bar and liquid
Lever 2000 bar and liquid
Irish Spring bar
Dial bar and liquid
Softsoap Antibacterial Soap

Antibiotic Ointments and Creams

Bacitracin
Polysporin
J & J First Aid Cream
Neomycin

Antibacterial Dishwashing Liquids

Dawn
Joy
Dial
Palmolive

Appendix E
HT-01-108589-11
CRB-01-04-063-HB

MICROBIOLOGICAL MEDIA AND METHODS

0.075M Phosphate Buffer Solution with Neutralizers

Weigh 0.4 grams of KH_2PO_4 , 10.1 grams of Na_2HPO_4 , 5.0 grams of Polysorbate (Tween) 80, 0.7 grams of lecithin, and 1.0 gram of Triton X-100. Dissolve in 1 liter of distilled or deionized water. Adjust to pH 7.9 ± 0.1 with 1 N HCl or 1 N NaOH. Dispense buffer in bottles so that after autoclaving the volume equals 75 ± 1 mL. Loosely cap bottles and sterilize in the autoclave at 121°C .

0.0375M Phosphate Buffer Solution with Neutralizers

Weigh 0.2 grams of KH_2PO_4 , 5.05 grams of Na_2HPO_4 , 5.0 grams of Polysorbate (Tween) 80 and 0.7 grams of lecithin. Dissolve in 1 liter of distilled or deionized water. Adjust to pH 7.9 ± 0.1 with 1 N HCl or 1 N NaOH. Dispense buffer in appropriate volumes. Loosely cap vessels and sterilize in the autoclave at 121°C .

MacConkey's Agar

Suspend 50 grams in 1 liter of distilled or deionized water. Loosely cap flask and sterilize in the autoclave at 121°C . Cool to $45\text{-}50^\circ\text{C}$ in a water bath. Pour in sterile 15 x 100 mm Petri dishes. Allow to cool and solidify on a level flat surface. Check for sterility. Prepared plates are stored at $2 - 8^\circ\text{C}$ and used within 30 days.

Estimated Plate Count Procedure

Do not record counts on crowded plates from the highest dilution as too numerous to count (TNTC). If the number of colonies per plate exceeds 250, count colonies in those portions of the plate that are representative of colony distribution and calculate the Estimated Standard Plate Count (ESPC) from these counts. The ESPC will be determined utilizing the grid embossed area on the lighted surface of the colony counter. Each large square on the grid is 1 cm^2 . If there are fewer than 10 colonies per square centimeter, count colonies in 12 squares, selecting, if representative, six consecutive squares horizontally across the plate and six consecutive squares at right angles, being careful not to count a square more than once. When there are more than 10 colonies per square centimeter, count colonies in four such representative portions. In both instances, multiply the average found per square centimeter by the area of the plate used to determine the estimated number of colonies per plate.

If the total number of CFU's have been estimated according to the procedure described above, ESPC (Estimated Standard Plate Count) should be recorded following the value.

Note: If the highest dilution plated contains >250 CFU's and a count ≤ 300 CFU's has been previously determined, that value may be reported. It will not be necessary to estimate the total CFU's on a plate containing >250 CFU's using the above procedure. Plates containing the highest dilution of test specimen plated and the CFU counts are greater than 300, then the above procedure should be used to determine the total CFU count.

Data Collection Form 1
DEMOGRAPHICS/DERMATOLOGICAL/MEDICAL HISTORY FORM

Study #	Hill Top Research, Inc.	Visit Code	Date	Subject Initials	Subject Screen #
01-108589-11 CRB-01-04-063-HB		Subject Qualification	____/____/____ mm dd yy	____/____/____ F M L	Permanent #:

Gender: <input type="checkbox"/> Male ⁽¹⁾ <input type="checkbox"/> Female ⁽²⁾	Age: _____ Years
--	--------------------------------

Does the subject have any of the following at the treatment sites?

I. DERMATOLOGIC DISORDER	No	Yes	Don't Know
1. Psoriasis ?			
2. Eczema ?			
3. Skin Cancer ?			
4. Skin Allergies ? Please specify:			
5. Hives ?			

Does the Subject have any of the following (present and past)?

II. OTHER MEDICAL INFORMATION	No	Yes	Don't Know
1. Allergies? Please specify.			
2. Hepatitis ?			
3. Heart and Vascular Disease?			
4. Liver Disease ?			
5. Kidney Disease ?			
6. Tuberculosis ?			
7. Diabetes ? Controlled? Diet [] Oral [] Insulin []			
8. Cancer ?			
9. Auto-immune disease (Lupus erythematosus, thyroiditis, AIDS, etc.) ?			
10. Organ transplant ?			
11. Any other condition not listed ? Please specify			

Is the subject taking any medication? If yes, please specify below:

III. MEDICATION	No	Yes	Don't Know
1. Antibiotics, oral or systemic ?			
2. Cortisone, Steroids, ACTH, Anti-reaction Drugs ?			
3. Heart Medication ?			
4. Insulin ?			
5. Other ?			

Comments:

Based on the above medical history, the subject is: ☐ **Qualified** or ☐ **Not qualified** for the study.

Interviewer's Signature:	Date: ____/____/____ mm dd yy
---------------------------------	---

Data Collection Form 2
INCLUSION / EXCLUSION FORM

Study #	Hill Top Research, Inc.		Visit Code	Date	Subject Initials	Subject Screen #
01-108589-11 CRB-01-04-063-HB			Subject Qualification	mm dd / yy	F / M / L	Permanent #:
INCLUSION CRITERIA						
Check one						
YES	NO	Subject:				
		1. Is ≥ 18 years ?				
		2. Has signed informed consent?				
		3. Is healthy as evidenced by responses on DCF 1 ?				
		4. Has hands and wrists that are free of dermatoses, cuts, lesions, and other skin disorders ?				
		5. Is willing to comply with all study protocol requirements ?				
EXCLUSION CRITERIA						
YES	NO	N/A	Subject:			
			1. Is currently participating in another clinical study at this or any other facility?			
			2. Has participated in any type of hand or arm wash study within the past 7 days?			
			3. Has cuts, lesions, or other skin disorders on their hands or wrists?			
			4. Has soap, detergent, antibiotic and/or perfume allergies?			
			5. Has eczema or psoriasis on their hands or wrists?			
			6. Has used antibacterial/antimicrobial soaps, medicated lotions and creams and/or anti-dandruff shampoos within the last week?			
			7. Has long (≥ 2 mm free edge) or artificial nails			
Female	Female	Male	8. Is currently pregnant ? <input type="checkbox"/> Yes <input type="checkbox"/> No Of child-bearing potential: <input type="checkbox"/> Yes <input type="checkbox"/> No <input type="checkbox"/> Surgically Sterile <input type="checkbox"/> Post-menopausal If of child bearing potential - β -HCG Test Results: <input type="checkbox"/> negative <input type="checkbox"/> positive			
Female	Female	Male	9. Is currently lactating?			
			10. Has been medically diagnosed as having a medical condition such as: diabetes, hepatitis, an organ transplant, or AIDS (or HIV positive) ?			
			11. Has another medical condition which in the opinion of the Investigator would preclude participation ?			
Based upon dermatologic evaluation and the information contained in Data Collection 1 and 2, the subject is:						
<input type="checkbox"/> Qualified <input type="checkbox"/> Not Qualified for participation in this study.						
Reasons for disqualification: _____						
Interviewer's Signature				Date: ____ / ____ / ____ mm dd yy		
Investigator's Signature::				Date: ____ / ____ / ____ mm dd yy		

Source Document 2
TREATMENT PHASE

Study #	Hill Top Research, Inc.	Permanent #'s
01-108589-11 CRB-01-04-063-HB		

EVENT	TIME	PROCEDURE PERFORMED ACCORDING TO PROTOCOL?	
Test Contamination Procedure #2	am/pm	<input type="checkbox"/> Yes / <input type="checkbox"/> No	
Test Product Treatment #2	am/pm	<input type="checkbox"/> Yes / <input type="checkbox"/> No	Water Temp: °F
Test Contamination Procedure #3	am/pm	<input type="checkbox"/> Yes / <input type="checkbox"/> No	
Test Product Treatment #3	am/pm	<input type="checkbox"/> Yes / <input type="checkbox"/> No	Water Temp: °F
Test Contamination Procedure #4	am/pm	<input type="checkbox"/> Yes / <input type="checkbox"/> No	
Test Product Treatment #4	am/pm	<input type="checkbox"/> Yes / <input type="checkbox"/> No	Water Temp: °F
Test Contamination Procedure #5	am/pm	<input type="checkbox"/> Yes / <input type="checkbox"/> No	
Test Product Treatment #5	am/pm	<input type="checkbox"/> Yes / <input type="checkbox"/> No	Water Temp: °F
Test Contamination Procedure #6	am/pm	<input type="checkbox"/> Yes / <input type="checkbox"/> No	
Test Product Treatment #6	am/pm	<input type="checkbox"/> Yes / <input type="checkbox"/> No	Water Temp: °F
Test Contamination Procedure #7	am/pm	<input type="checkbox"/> Yes / <input type="checkbox"/> No	
Test Product Treatment #7	am/pm	<input type="checkbox"/> Yes / <input type="checkbox"/> No	Water Temp: °F
Test Contamination Procedure #8	am/pm	<input type="checkbox"/> Yes / <input type="checkbox"/> No	
Recorder's Signature:		Date: ____/____/____	
Reviewer's Signature:		Date: ____/____/____	

Source Document 2 (continued)

TREATMENT PHASE

Study #	Hill Top Research, Inc.			Permanent #'s
01-108589-11 CRB-01-04-063-IIB				

Test Product Treatment #8	am/pm	<input type="checkbox"/> Yes / <input type="checkbox"/> No	Water Temp:	°F
Test Contamination Procedure #9	am/pm	<input type="checkbox"/> Yes / <input type="checkbox"/> No		
Test Product Treatment #9	am/pm	<input type="checkbox"/> Yes / <input type="checkbox"/> No	Water Temp:	°F
Recorder's Signature:		Date: ____ / ____ / ____		
Reviewer's Signature:		Date: ____ / ____ / ____		

Source Document 1
TREATMENT PHASE

Study #	Hill Top Research, Inc.		Permanent #'s
01-108589-11 CRB-01-04-063-HB			

EVENT	TIME	PROCEDURE PERFORMED ACCORDING TO PROTOCOL?
Practice Wipe	am/pm	<input type="checkbox"/> Yes / <input type="checkbox"/> No
Decontamination	am/pm	<input type="checkbox"/> Yes / <input type="checkbox"/> No
Base Contamination Procedure	am/pm	<input type="checkbox"/> Yes / <input type="checkbox"/> No
Base Bacterial Sampling Procedure	am/pm	<input type="checkbox"/> Yes / <input type="checkbox"/> No
Base Decontamination	am/pm	<input type="checkbox"/> Yes / <input type="checkbox"/> No
Test Contamination Procedure #1	am/pm	<input type="checkbox"/> Yes / <input type="checkbox"/> No
Test Product Treatment #1	am/pm	<input type="checkbox"/> Yes / <input type="checkbox"/> No
		Water Temp: °F
Test Bacterial Sampling Procedure #1 (after first treatment)	am/pm	<input type="checkbox"/> Yes / <input type="checkbox"/> No
Test Contamination Procedure #10	am/pm	<input type="checkbox"/> Yes / <input type="checkbox"/> No
Test Product Treatment #10	am/pm	<input type="checkbox"/> Yes / <input type="checkbox"/> No
		Water Temp: °F
Test Bacterial Sampling Procedure #2 (after 10th treatment)	am/pm	<input type="checkbox"/> Yes / <input type="checkbox"/> No
Decontamination	am/pm	<input type="checkbox"/> Yes / <input type="checkbox"/> No
		Water Temp: °F

Recorder's Signature:	Date: ____/____/____
Reviewer's Signature:	Date: ____/____/____

Data Collection Form 3

FOLLOW-UP VISIT

Study #	Hill Top Research, Inc.	Visit Code	Date	Subject Initials	Subject Screen #
01-108589-11 CRB-01-04-063-HB		Follow-up	mm / dd / yy	F / M / L	Permanent #:

Date Subject Entered the Study: mm / dd / yy	Follow-up Visit Date : mm / dd / yy
<p>Does the subject's hands have the presence of pimples, blisters, or raised itching bumps surrounded by erythema and/or edema that may be indicative of a skin infection ?</p> <p>YES NO If yes, complete below:</p> <p>Clinical Observations: (Include date of onset and descriptions severity locations, etc.)</p> <p>_____</p> <p>_____</p> <p>Comments: _____</p> <p>_____</p> <p>_____</p> <p>_____</p> <p>_____</p>	
<p>Has the subject had any health related issues since the treatment procedure?</p> <p>YES NO If yes, complete below:</p> <p>Comments: _____</p> <p>_____</p> <p>_____</p> <p>_____</p> <p>_____</p> <p>_____</p>	

Investigator's Signature or designee	Date mm / dd / yy
--------------------------------------	----------------------

Data Collection Form 4

ADVERSE EVENT

Study #	Hill Top Research, Inc.		Date	Subject Initials	Subject Screen #
01-108589-11 CRB-01-04-063-HB			mm / dd / yy	F / M / L	Permanent #:

Was reaction related to treatment? ☐ Not related ☐ Possibly related ☐ Definitely related ☐ Other (explain)

Did subject take any medication during the study period? ☐ YES ☐ NO If yes, complete section below.

Date of Onset: _____ Date Reported: _____ Date Resolved: _____

Describe event: _____

Action Taken: ☐ None ☐ Continued on study ☐ Withdrawn from the study ☐ Consulted physician

☐ Medication taken (Complete below) ☐ Hospitalized ☐ Other (explain)

Additional Comments:

FOLLOW - UP ACTION TAKEN

Date	Action Taken	Comments	Initials

CONCOMITANT MEDICATION TAKEN

Medication (Oral or Systemic)	Total Daily Dose	Start Date mm dd yy	Stop Date mm / dd / yy	Indication (Reason for Taking)
			/ /	
			/ /	
			/ /	

Investigator's Signature:

Recorded by:

Date

mm / dd / yy

Data Collection Form 5

PHYSICIAN'S ACTION REPORTING FORM

Study #	Hill Top Research, Inc.		Date	Subject Initials	Subject Screen #
01-108589-11 CRB-01-04-063-HB			mm / dd / yy	F / M / L	Permanent #:

Date(s) of office visit(s): _____

Pertinent Medical History: (e.g., causes of similar reactions, known allergies,
potential involvement of current medications or medical conditions)

Test Product Exposure:

Use Began On: _____ Date Used Ended on: _____ Date Number of Uses: _____

Clinical Observations: (Include date of onset and descriptions severity locations, etc.)

Impression: _____

Treatment: _____

Follow Up: _____

Date Resolved: _____

Is condition related to use of the test products?

☐ Probably related*

☐ Not Related*

☐ Unknown

Reasons: _____

Physician's Signature

Date

CRB-01-04-063-HB

[illegible]

Institution: Hill Top Research, Inc.
Investigator: Gayle K. Mulberry, M.S.

HTR Study No. 01-108589-11
Sponsor No. CRB 01-04-063-HB
Page No. II-14

Study Title: "Efficacy Evaluation of Three Handwipe Products In a Modified Health Care Personnel Handwash Study Versus *Escherichia coli*"

CONSENT FORM

INTRODUCTION: You are being asked to take part in a research study. Before you give your consent to be a subject, it is important that you take enough time to read and understand what your participation would involve. In preparing this consent form, it has been necessary to use some technical language. Please ask questions if there is anything you do not understand.

You will be given a signed copy of this consent form and any other necessary written information prior to the start of the study.

PURPOSE: The purpose of this research study is to determine the effectiveness of three handwipe products containing an antibacterial ingredient against bacteria found on the skin. Approximately twenty (20) people at least 18 years of age will be screened as potential subjects in this study. At least twelve (12) subjects are expected to complete the two-visit study.

TEST ARTICLES: You will be assigned 1 of the three antibacterial handwipe products. The handwipe products are experimental.

STUDY PROCEDURES: Prior to enrollment in the test, you will be asked to complete a brief medical history questionnaire and another form to determine your eligibility for the study. Your hands and wrists will be checked for visible cuts, scratches or rashes on them. It is possible that you may not be able to participate based on your answers to these questions or the condition of the skin on your hands and wrists.

If you are selected to participate in this study, you will be instructed to perform a practice treatment with a handwipe product. Then, your hands will be rinsed with alcohol, rubbed for about 15 seconds and rinsed in tap water for 15 seconds followed by drying with paper towels. Afterwards, your hands will be contaminated with a watery liquid containing relatively non-harmful bacteria (*Escherichia coli*). This liquid containing the bacteria will be spread over the surfaces of the hands, and the hands will be allowed to air dry. Following air drying, the hands will be sampled. Sampling is accomplished by having you place your hands into large plastic bags to which will be added a mild soap-like solution. A laboratory technician will massage each bagged hand for one minute. The hands will be removed from the bags and the solution from each bag will be tested to determine the number of test bacteria added to the hands. Following this baseline sampling, the hands will be rinsed for 30 seconds with tap water, rinsed with 70% alcohol and water, then dried with paper towels. Then the hands will be contaminated as above and treated with the assigned test material, 1

of the 3 handwipe products following specific directions. After the treatment with the handwipe product, your hands will be sampled as above about 4-5 minutes after the first treatment is completed to determine the number of bacteria removed or killed by treatment. Your hands will be contaminated and treated 10 times. After the 10th treatment, sampling will be repeated. Following each sampling, your hands will be rinsed with tap water. After the final sampling your hands will then be washed with a plain soap followed by a wash with Hibiclens®, an antimicrobial soap, and rinsed with alcohol prior to leaving the lab.

After completing the treatment visit and until your follow-up visit, you will need to check the skin on your hands each day for any pimples, bumps or rashes. Within four to nine days after you have completed treatment, you will be required to return to the lab for a follow-up visit. Your hands will be checked for infection by a technician trained in observing infection.

FEMALES OF CHILDBEARING POTENTIAL: You may not participate in this study if you are pregnant or nursing. As part of giving your consent you must agree to have a urine pregnancy test at the start of the study.

RISKS: The risks associated with this test are primarily related to infection with the test bacteria. For healthy persons, the possibility of a skin infection exists; however, this possibility is remote because, (1) test bacteria are applied only to healthy or uninjured skin, and (2) the skin is cleansed with antibacterial products following contact with the test bacteria. Your hands may also show a "reaction." A "reaction" could be pimples, blisters or raised bumps surrounded by redness and/or swelling. It is unlikely, but possible, that a rash could develop.

No risks to you as a study participant, other than those described above as "reactions," are anticipated during the study. Reactions are usually due to irritation, although an allergic reaction might occur. If you become allergic, it is possible that future exposures to the same ingredient may cause a skin reaction. If this occurs, you will be provided with information to minimize the chance for future exposures.

You may experience risks or side effects that are not known at this time. You will be informed in a timely manner if new information becomes available that may influence your willingness to continue in this study.

BENEFITS: You will not benefit from the application of test product but the study results may allow a new or improved product to be marketed.

ALTERNATIVE PROCEDURES/TREATMENTS: Because you are not being treated for a medical condition, alternative treatments do not apply to this study.

CONFIDENTIALITY: Information concerning you that is obtained in connection with this study will be kept confidential by Hill Top Research, except that the sponsoring company whose product is being tested will receive a copy of the study records. The records will be coded to protect your identity. In addition, government regulatory agencies, including the U.S. Food and Drug Administration (FDA), may inspect the records of the study. Information obtained in the study may be used for medical or scientific publication, but your identity will remain confidential.

MEDICAL TREATMENT: If in the course of this study you experience illness, discomfort or injury that appears to be a result of the study, Hill Top Research will provide you with medical care at no cost to you. Providing such medical care is not an admission of legal responsibility. If such illness, discomfort or injury does occur, ask any staff member to arrange a meeting for you with the appropriate personnel.

In certain cases of illness or injury resulting from this study, workers' compensation coverage may be available. In accordance with Ohio law, Hill Top Research has secured workers' compensation coverage for participants in its studies and tests, and has paid and will pay appropriate premiums into the State Insurance Fund on behalf of such participants.

WHO TO CONTACT: If you have any questions about this study or in case of an emergency, contact Stacey, Study Coordinator at 513-831-3114, ext. 2324 during business hours (M-F, 8:00 A.M. - 5:00 P.M.) or Ann Brady, Study Manager at 513-831-3354 after hours.

VOLUNTARY PARTICIPATION/WITHDRAWAL: Your participation in this research study is strictly voluntary. You may refuse to participate or may discontinue participation at any time during the study without penalty or loss of benefits to which you are otherwise entitled.

If you agree to participate in this study, you are also agreeing to provide Hill Top Research with accurate information and to follow study instructions as given to you. If you fail to comply with study procedures, your participation may be terminated.

Your participation in the study may be discontinued at any time without your consent by the Investigator, the FDA, or the sponsoring company.

COMPENSATION: You will be paid \$55.00 for the completion of this study. You will be compensated according to the following schedule:

If you do not qualify	Visit 1	you will receive	\$10.00
If you qualify but are eliminated as an extra subject	Visit 1	you will receive	\$15.00
If you complete	Visit 1	you will receive	\$30.00
If you complete	Visit 2	you will receive	\$55.00

Payments will be made at the end of the study.

There are no anticipated expenses to you for participating in this study. All test related materials will be provided at no cost to you.

CONSENT TO PARTICIPATE

I know that my participation in this study is voluntary and that I have the right to refuse to participate. I know that I may withdraw from the study at any time without penalty or loss of benefits to which I am otherwise entitled. If I withdraw or am dismissed for failure to obey rules or follow directions, I understand I will only be paid for the portion of the study that I have completed. If, in the judgment of the Investigator, it is best to discontinue my participation in the study for other reasons, I will be paid either in full or for that portion of the study already completed.

If I am a female of childbearing potential, I am not currently pregnant or nursing an infant. I am using an adequate means of birth control and, if I become pregnant or believe I have become pregnant, I will notify the Investigator immediately.

CONSENT: I have read all of the above information and have been given an opportunity to ask questions about this study. Answers to such questions (if any) were satisfactory. I am eighteen years of age or older and freely and without reservation give my consent to serve as a subject in this study. By signing this form, I have not given up any of my legal rights as a research subject.

Subject's Name Printed: First

Middle Initial

Last

Subject's Signature

Date

Signature of Person Conducting Consent Discussion

Date

SUBJECT SCREEN NO. _____

SUBJECT NO. _____

F

FINAL REPORT OF CLINICAL TEST RESULTS

From: P. B. Neumann, J. S. Englehart

Date: July 10, 1998

To: Study File for CRB-97-11-245-CD

Retention Limit: Until Superseded

Released: WLB 7/10/98

Subject: Residual Effectiveness Screening Test Results on B-22M Liquid Soap Formulations with Varying Levels of Salicylic Acid and Triclosan (TCS) and Liquid Ivory® against *E. coli* - Hill Top Research, Inc., CRB-97-11-245-CD, HT 97-5425-11

Summary:

The results of this clinical study show that B-22M liquid soap formulations with either no TCS, 0.5% TCS or 1.0% TCS and no salicylic acid (codes A, B and C, respectfully) were not significantly more effective than Liquid Ivory, code J, at lowering the levels of *Escherichia coli* inoculated on the skin (p-value <0.10). All other B-22M liquid soap formulations with either 0.5% or 1.0% salicylic acid and varying levels of TCS, ranging from none to 1.0%, were significantly more effective than Liquid Ivory at lowering organisms inoculated on the skin.

The results are summarized in the attached tables.

Objective:

The objective of this study was to evaluate the residual effectiveness of ten (10) liquid soap products against potentially pathogenic bacteria (*Escherichia coli*, ATCC 11229) under simulated skin conditions which are considered optimal for bacterial growth, proliferation, and possible infection.

Test Products:

Code	Test Product	Active Ingredient	TSIN
A	B-22M	none	BI0060-108
B	B-22M	0.5% TCS	BI0060-110
C	B-22M	1.0% TCS	BI0060-112
D	B-22M, 0.5% salicylic acid	none	BI0060-100
E	B-22M, 0.5% salicylic acid	0.5% TCS	BI0060-102
F	B-22M, 0.5% salicylic acid	1.0% TCS	BI0060-104
G	B-22M, 1.0% salicylic acid	none	BI0060-092
H	B-22M, 1.0% salicylic acid	0.5% TCS	BI0060-094
I	B-22M, 1.0% salicylic acid	1.0% TCS	BI0060-096
J	Liquid Ivory Soap	none	BI0060-126

Study Design:

This study was conducted at Hill Top Research, Inc. in Miamiville, Ohio on December 1, 1997. This was a randomized, split forearm wash study to evaluate the residual antibacterial effectiveness of ten (10) liquid products. Thirty (30) male and female subjects, ages 18 through 65 years old, who did not regularly use

Study Design: (continued)

antibacterial soap, medicated lotion or cream, and/or antidandruff shampoo were enrolled. For the test procedure, each of the subject's forearms was divided into an upper and lower treatment area, for a total of four (4) treatment areas per subject. Each treatment area was washed one (1) time for forty-five (45) seconds. Within five (5) minutes after the wash with the test products was completed, a 3.0 cm circular test site was marked-off in each area. Each circular site was then inoculated with 10 μ L of a 24 hour broth culture of *E. coli* grown in Trypticase Soy Broth (TSB) and occluded with a 24 mm Hill Top Chamber® that was taped to the skin with Durapore® tape. One (1) hour after inoculation, the surviving organisms were harvested from each occluded site using the Williamson-Kligman scrub technique. The specimens from each site were plated on Trypticase Soy Agar with polysorbate (Tween) 80 and incubated for 18 - 24 hours at $35 \pm 2^\circ\text{C}$. The colony forming units (CFU's) of *E. coli* were counted at the end of the incubation period to determine the number of surviving organisms at each of the treated sites.

Data Analysis:

The surviving colony forming units (CFU's) of bacteria for each subject were enumerated. The numbers of bacteria were converted to base 10 logarithms. The \log_{10} CFU counts were compared using analysis of variance techniques. Factors were treatment levels and subject, adjusting for subject to subject variability, side (right vs. left) variability, site (upper vs. lower) variability, and site to side variability, to estimate which of the test products had the greatest activity. P-values ≤ 0.10 were considered statistically significant.

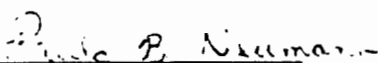
Subject Accountability:

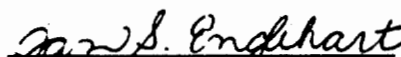
Thirty (30) subjects were enrolled and completed this study.

Adverse Events:

None reported.

Submitted by:


Paula B. Neumann


Jan S. Englehart

Conclusions:

The three B22M prototypes with no salicylic acid were not significantly more effective than Liquid Ivory Soap. All other prototypes were significantly more effective against *E. coli* at p-values ≤ 0.10 .

Salicylic acid was a significant factor (p-value = 0.0044) in efficacy against *E. coli*, whereas TCS was not (p-value = 0.4652).

Both the 0.5% and 1% salicylic acid B22M prototypes were significantly more effective than Liquid Ivory Soap even when TCS was not present at p-values ≤ 0.10 .

Although the interaction between TCS and salicylic acid was not significant, (p-value = 0.1386) it was close enough to significance to merit examination. It appears as though some salicylic acid was necessary to provide a dose response with TCS in the ranges examined in this study and that this response is greater than 0.5% salicylic acid. See Figures 1 & 2.

Treatment	Code	N	Mean	Std. Dev.
B22M, 0.0% TCS, 0.0% Salicylic Acid	A	12	4.9	0.546
B22M, 0.5% TCS, 0.0% Salicylic Acid	B	12	5.2	0.524
B22M, 1.0% TCS, 0.0% Salicylic Acid	C	12	5.0	0.510
B22M, 0.0% TCS, 0.5% Salicylic Acid	D	12	4.3	1.086
B22M, 0.5% TCS, 0.5% Salicylic Acid	E	12	4.1	1.292
B22M, 1.0% TCS, 0.5% Salicylic Acid	F	12	4.0	0.967
B22M, 0.0% TCS, 1.0% Salicylic Acid	G	12	4.2	0.713
B22M, 0.5% TCS, 1.0% Salicylic Acid	H	12	3.8	0.936
B22M, 1.0% TCS, 1.0% Salicylic Acid	I	12	3.5	1.411
Liquid Ivory Soap	J	12	5.3	0.6074

One Way Layout Ignoring DOX Design:

Treatment	LSMean	Error
A	4.7	0.220
B	4.9	0.220
C	5.0	0.220
D	4.4	0.220
E	4.4	0.220
F	4.3	0.220
G	4.3	0.220
H	3.9	0.220
I	3.4	0.220
J	5.1	0.220

Overall P-value=0.0001

Pairwise P-values (One Way Layout Ignoring DOX Design)										
i/j	A	B	C	D	E	F	G	H	I	J
A	.	0.4224	0.2858	0.3409	0.3661	0.3463	0.3438	0.0234	0.0001	0.1145
B	0.4224	.	0.7357	0.0793	0.0997	0.1066	0.1109	0.0052	0.0001	0.4608
C	0.2858	0.7357	.	0.0284	0.0396	0.0474	0.0595	0.0025	0.0001	0.6923
D	0.3409	0.0793	0.0284	.	0.9976	0.9598	0.9659	0.2017	0.0071	0.0273
E	0.3661	0.0997	0.0396	0.9976	.	0.9548	0.9614	0.1790	0.0065	0.0290
F	0.3463	0.1066	0.0474	0.9598	0.9548	.	0.9359	0.1681	0.0050	0.0245
G	0.3438	0.1109	0.0595	0.9659	0.9614	0.9959	.	0.1409	0.0029	0.0184
H	0.0234	0.0052	0.0025	0.2017	0.1790	0.1681	0.1409	.	0.0766	0.0002
I	0.0001	0.0001	0.0001	0.0071	0.0065	0.0050	0.0029	0.0766	.	0.0001
J	0.1145	0.4608	0.6923	0.0273	0.0290	0.0245	0.0184	0.0002	0.0001	.

NOTE: To ensure overall protection level, only probabilities associated with pre-planned comparisons should be used.

Salicylic Acid	LSMean	Std. Error	Pairwise P-values		
			1	2	3
1 = 0.0%	4.8	0.162	1	0.0428	0.0011
2 = 0.5%	4.3	0.152	2	0.0428	0.0618
3 = 1.0%	3.9	0.162	3	0.0011	0.0618

Overall P-value = 0.0044

TCS	LSMean	Std. Error	Pairwise P-values		
			1	2	3
1 = 0.0%	4.5	0.120	1	0.7083	0.2287
2 = 0.5%	4.4	0.120	2	0.7083	0.4029
3 = 1.0%	4.2	0.120	3	0.2287	0.4029

Overall P-value = 0.4652

Salicylic Acid	TCS	LSMean	Std. Error
1 = 0.0%	0.0%	4.6	0.239
2 = 0.0%	0.5%	4.9	0.244
3 = 0.0%	1.0%	5.0	0.242
4 = 0.5%	0.0%	4.4	0.235
5 = 0.5%	0.5%	4.3	0.234
6 = 0.5%	1.0%	4.3	0.235
7 = 1.0%	0.0%	4.4	0.242
8 = 1.0%	0.5%	3.9	0.244
9 = 1.0%	1.0%	3.4	0.239

Overall P-value = 0.1386

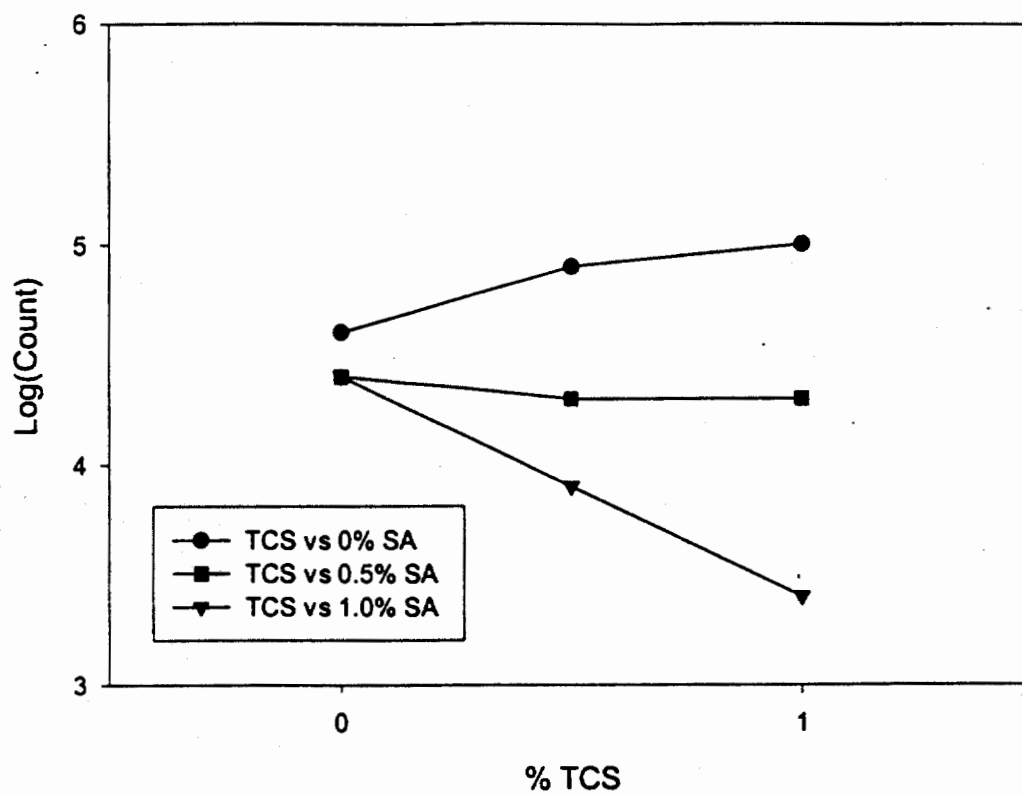
Pairwise P-values:

i/j	1	2	3	4	5	6	7	8	9
1		0.4355	0.3262	0.3871	0.4214	0.4118	0.4429	0.0484	0.0005
2	0.4355		0.7861	0.1022	0.1317	0.1468	0.1739	0.0148	0.0001
3	0.3262	0.7861		0.0450	0.0638	0.0791	0.1107	0.0084	0.0001
4	0.3871	0.1022	0.0450		0.9861	0.9846	0.9809	0.2544	0.0150
5	0.4214	0.1317	0.0638	0.9861		0.9695	0.9923	0.2207	0.0128
6	0.4118	0.1468	0.0791	0.9846	0.9695		0.9614	0.2003	0.0095
7	0.4429	0.1739	0.1107	0.9809	0.9923	0.9614		0.1559	0.0053
8	0.0484	0.0148	0.0084	0.2544	0.2207	0.2003	0.1559		0.1046
9	0.0005	0.0001	0.0001	0.0150	0.0128	0.0095	0.0053	0.1046	

NOTE: To ensure overall protection level, only probabilities associated with pre-planned comparisons should be used.

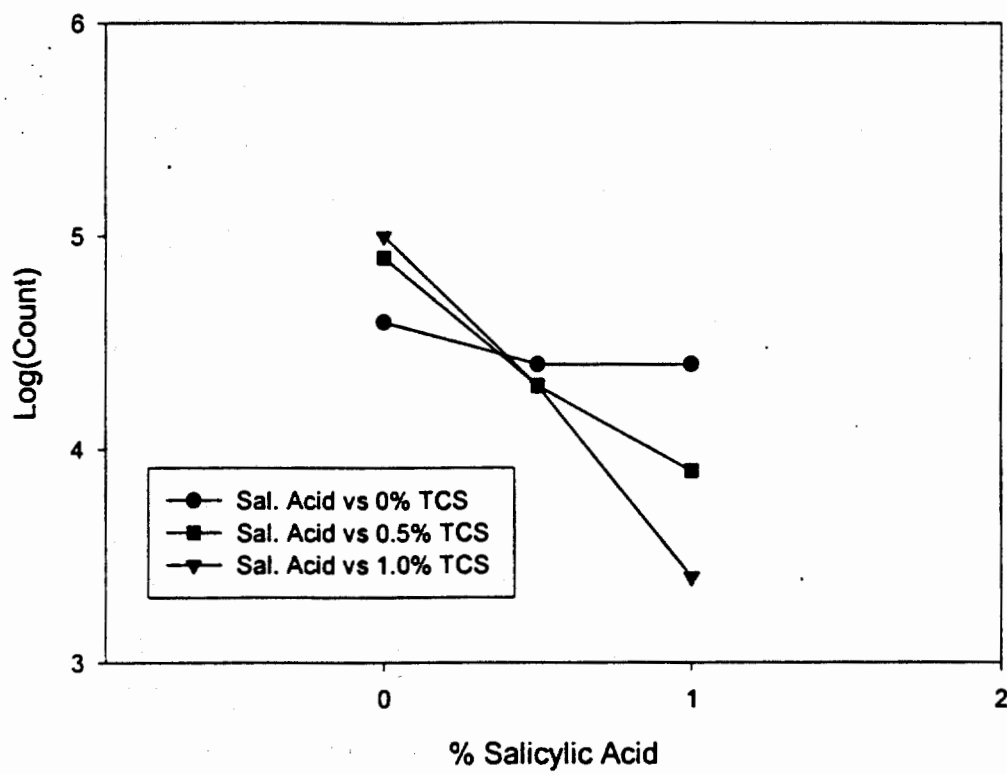
P. B. Neumann

Figure 1 - Plot of Log(Count) by %TCS and % Salicylic Acid



P. B. Neumann

Figure 2 - Plot of Log(Count) by % TCS and % Salicylic Acid



P. B. Neumann

**RESIDUAL EFFECTIVENESS SCREENING TEST OF
ANTIBACTERIAL LIQUID PRODUCTS AGAINST *E. COLI***

Study Number: CRB-97-11-245-CD

Study Identification: HT# 97-5425-11

Principal Investigator: Gayle K. Mulberry, M.S.

Sub-Investigator: Ann R. Brady, A.S.

Clinical Test Site: Hill Top Research, Inc.
Main and Mill Streets
Miamiaville, Ohio 45147

Sponsor: The Procter & Gamble Company
Clinical Research and Biometrics Division
Personal Cleansing Sector
11511 Reed Hartman Highway
Cincinnati, Ohio 45241

Sponsor Representative: Ward L. Billhimer, M.S.

Sponsor Statistician: Paula B. Neumann, Ph.D.

Sponsor Toxicologists: Paul F. Sterchele, Ph.D.
J. David Innis, Ph.D.

Clinical Research Associates: Jan S. Englehart, B.S., ASCP

Start Date: December 1, 1997

Confidentiality: The obligations of the Investigator, regarding the confidential information on the antibacterial soap and all aspects of the study will be kept confidential according to the agreement with The Procter & Gamble Company.

I. Study Objective

The objective of this study is to evaluate the residual effectiveness of ten (10) liquid soap products containing an antibacterial active against potentially pathogenic bacteria (*Escherichia coli*, ATCC 11229) under simulated skin conditions which are considered optimal for bacterial growth, proliferation, and possible infection.

II. Study Summary

This is a randomized blinded clinical study that will evaluate the residual effectiveness of ten (10) liquid soap products using a split forearm test design. It will consist of a one (1) day test period. Thirty (30) male and female subjects, age 18 to 65 years, who do not regularly use antibacterial/antimicrobial soaps, medicated lotions and creams, and/or anti-dandruff shampoos will be enrolled into the study. During this study, thirty (30) subjects will be used to evaluate ten (10) test products.

On the day of the test, subjects will report to the clinical test facility. Each of the subjects' forearms will be divided into an upper and lower treatment area for a total of four (4) treatment areas. Subjects will have each of their forearms washed by a laboratory technician with the test products according to a randomization.

Following treatment with the test products, a test site will be marked off in the center of each treatment area on the forearms. Each of the four (4) test sites will be inoculated with a known amount of *Escherichia coli* (ATCC 11229) grown in Trypticase Soy Broth (TSB). The test sites will then be occluded with a Hill Top Chamber® patch for one (1) hour. After occlusion, the patches will be removed and the bacteria on the skin will be harvested using a scrub technique ⁽¹⁾. Each sample of harvested bacteria will be diluted, plated, and incubated. Following incubation, the number of surviving colony forming units (CFU's) for each site will be determined.

III. Study Population

Subjects will be screened for their eligibility to participate based upon information provided in the Demographics/Dermatological/Medical History Form [Case Report Form (CRF) 1]. Only subjects meeting the inclusion/exclusion criteria, outlined in CRF 2, will be allowed to participate in the study. If a subject is admitted to this study in apparent violation of any of the above criteria, the reason(s) for admission will be noted by the Investigator or his designee.

A. Subject Inclusion Criteria

Subjects are eligible for enrollment if they

1. Are a male or female, age 18 to 65 years;
2. Have signed the Informed Consent (Appendix A);
3. Are in good health, as evidenced by response to the Demographics/ Dermatological/Medical History Form (CRF 1);
4. Have forearms that are free of dermatoses, cuts, lesions, and other skin disorders; and
5. Are willing to comply with all study protocol requirements.

B. Subject Exclusion Criteria

A subject cannot be enrolled in the study if they:

1. Are currently participating in another clinical study at this or any other facility;
2. Have participated in any type of hand or arm wash study within the past 14 days;
3. Have cuts, scratches, or a rash on the volar surface of either forearm;
4. Have soap, detergent, and/or perfume allergies;
5. Have eczema or psoriasis on their arm(s);
6. Have taken systemic antibiotics or used topical antibiotics for any reason in the three (3) weeks prior to the start of the study;
7. Are currently using antibacterial/antimicrobial soaps (liquids and/or bars), medicated lotions and creams, and/or anti-dandruff shampoos;
8. Are currently pregnant;
9. Are currently lactating;
10. Have been medically diagnosed as having a medical condition which would preclude participation such as: diabetes, hepatitis, an organ transplant, or AIDS (or HIV positive); and/or
11. Have any other medical condition, which in the opinion of the Investigator would preclude participation.

IV. Study Design and Procedures

A. Randomization

The Sponsor will generate the study randomization for the assignment of treatment products. Each subject will be assigned to a treatment number which will become their permanent subject identification number. All subjects and site personnel, including the Investigator, will remain blinded to product identities.

B. Study Schedule

On the day of the test, prospective subjects will visit the test facility to complete a written informed consent, (Appendix A), the Demographics/Dermatological/ Medical History Form (CRF 1), and the Inclusion/Exclusion Form (CRF 2). Subjects who meet the study criteria, will be randomly assigned to a treatment regimen. Two (2) treatment areas, upper and lower, will be marked off on each arm. A lab technician will wash each of the treatment areas with one (1) of the test products according to the procedure described in Appendix C. After the wash with the test products is completed, a test site will be marked-off in the center of each treatment area on the subjects' forearms. These sites will be inoculated, occluded, and harvested according to the procedures described below.

To ensure that any delayed adverse events, primarily skin infections, are reported to the study Investigator, all test subjects will be given a copy of Subjects' Instructions Following Study Completion (Appendix B) before leaving the clinical test facility after they have completed the study. This sheet will instruct the subjects to examine their forearms 48 - 72 hours after completion of the study for the presence of pimples, blisters, or raised red itching bumps surrounded by erythema and/or

edema that may be indicative of a skin infection. Subjects, who notice any of these lesions, will be instructed to call the clinical test site.

C. Wash with the Test Products

Two (2) 10 x 5 cm treatment areas (upper and lower) will be marked off on each subject's forearms using a template. Each of the four (4) treatment areas on the subjects' forearms will be washed by a laboratory technician according to the procedure outlined in Wash Procedure, Appendix C. Each treatment area will be washed one (1) time with the appropriate test product. A record of the subject washes will be kept on CRF 3. In general, the following should be noted: water temperature should be closely monitored and maintained at 95-100°F. The water temperature should be recorded. Wash time should be recorded at the start of washing.

D. Microbial Inoculation

Within five (5) minutes after the wash with the test products is completed, one (1) circular test site will be marked-off in each treatment area of the subject's forearms. These circular sites will be spaced in the center of each treatment area of the forearm. They will be made by pressing a 3.0 cm diameter glass cylinder, inked with a stamp pad, against the skin.

All test sites on both arms will be inoculated with *E. coli* (ATCC 11229) that has been grown in Trypticase Soy Broth (TSB). To determine the actual number of CFU/mL at the time of inoculation, the broth culture to be used for inoculation will be plated.

Using an Eppendorf® pipette, the skin area delineated by the cylinder will be inoculated with 10 µL of the bacterial culture to obtain 10^6 to 10^7 colony forming units (CFU's) of *E. coli*. A sterile, disposable, inoculating loop will be used to evenly spread the inoculum within the center of the test site while remaining 4 to 5 mm from the marked edge. Inoculation of each site will be documented on CRF 3.

E. Occlusion of the Test Sites

The inoculated test site will be immediately occluded by covering it with a small plastic bowl (25 mm Hill Top Chamber® with pad removed) that will be secured to the skin with an adhesive dressing (Durapore®, 3M). The time of occlusion will be recorded on CRF 3.

F. Harvesting of the Surviving *E. coli* Organisms

All inoculated sites will be harvested for surviving organisms at 1 hour \pm 5 minutes after inoculation. The time of harvesting will be recorded on CRF 3.

The following procedure will be used for harvesting:

1. A hollow glass cylinder 2.2 cm in diameter will be positioned in the middle area of the test site avoiding contact with the ink-stamped edge.
2. 1.0 mL of phosphate-buffered 0.1% Triton X-100 detergent (pH 7.9), with suitable neutralizers, will be pipetted into the cylinder.
3. The skin inside the cylinder will be massaged for 60 seconds with a Teflon policeman.
4. The fluid will be removed by pipetting it into an empty sterile culture tube.
5. Another 1.0 mL of buffered detergent will be added for a second 30 second scrub.

6. The fluid from the second scrub will be removed and pooled with the fluid from the first scrub.

G. Disinfection of the Test Sites

After each test site is harvested, it will be disinfected with 70% isopropyl alcohol. When the harvesting of the last test site is completed, both forearms will be washed for approximately thirty (30) seconds with Hibiclens® (4% chlorhexidine gluconate). After the arms have been washed with Hibiclens®, a small amount of Polysporin® antibiotic ointment will be applied to each test site.

H. Plating and Incubation of the Organisms

Specimens from each of the four (4) sites will be plated within four (4) hours after harvesting. For plating, they will be serially diluted in half-strength (0.0375 M) buffer in ten-fold dilutions to 10^{-4} . 0.1 mL aliquots of each undiluted and diluted specimen will be pipetted onto the surface of duplicate plates, containing Trypticase Soy Agar with polysorbate (Tween) 80. The aliquots will be evenly spread on the surface of the plate with a sterilized bent glass rod. The media for these analyses are shown in Appendix F.

Plated samples will be incubated aerobically for 18 - 24 hours at $35 \pm 2^{\circ}\text{C}$. The CFU's of test bacteria will be counted at the end of the incubation period. In general, the number of CFU's per sample will be determined by taking the average of the counts from the plates which are in the range of ≥ 25 to ≤ 250 CFU's. If there are no plates with counts within this range, the following rules will be used to determine which counts will be used for the obtaining the number of CFU's for that specimen:

1. If all of the counts are below the prescribed range, the numbers below 25 from the undiluted plates will be used.
2. If the counts from the highest dilution are > 250 , the numbers, obtained from using the estimated counting procedure described in Appendix F, will be used.

The number of CFU's for each dilution counted will be recorded on Source Document 2.

V. Study Material and Instructions

A. Study Materials

All test products will be sent by the Sponsor to the clinical site prior to study initiation.

Each treatment product will be identified with the appropriate label (Appendix D) affixed to the outside of each container.

B. Shipping of Study Materials

The quantity of all materials, including test products and study supplies shipped to and returned from the clinical site, will be documented on the Shipping and Receiving Form (Source Document 1). The products will be packed into one or more cartons labeled with:

1. the study number;
2. distributor statement (i.e., "Distributed by Hill Top Research, Inc. with the test facility's full address and phone number); and
3. any applicable safety and handling procedures.

C. Return of Study Materials

Upon completion of the study, the Investigator will insure that all test products, whether completely used, partially used, or unused will be returned to the Sponsor at the following address:

The Procter & Gamble Company
Sharon Woods Technical Center
11511 Reed Hartman Highway
Cincinnati, Ohio 45241
Attn.: Jan Englehart

VI. Other Study Documentation and Requirements

A. Adverse Event and Intercurrent Event Reporting

Should any unexpected or serious adverse event occur during the clinical study or as a result of application of the test organism to the skin of the subjects, the subject will be requested to return to the site to be examined by the Investigator. The Investigator will determine whether: (a) the adverse event is likely to be associated with product treatment or the study procedures; (b) the event warrants termination of participation; and (c) to prescribe treatment, if necessary. The Investigator will notify the Sponsor representatives, Ward Billhimer, 513-626-1926 (work) or 513-831-8163 (home) or Jan Englehart, 513-626-1896 (work) or 513-385-9596 (home).

Each subject will need to be followed until the resolution of any adverse event. Information pertaining to the presenting signs, working diagnosis, assessment of the relationship of the adverse event to the product treatment, results of the follow-up visits and any prescribed treatment, will be documented in CRF 4. If treatment by a physician is necessary, this treatment will be documented on CRF 5.

B. Deviations from Protocol

Any deviations from the protocol that occur during execution and not previously agreed to by the Sponsor and Investigator will be documented. All changes in the protocol must be made in written amendments agreed upon by the Investigator and Sponsor. The amendments must be attached to the protocol on file.

C. Subject Termination and Completion

At the termination of the study, CRF 6 will be completed on all subjects. A concerted effort will be made to retain and follow all subjects in the study. Subjects, who terminate their own participation, prior to study closure, for any of the following reasons will also be documented in CRF 6.

- a) Intolerance of the study procedures.
- b) Intercurrent illness which interferes with the evaluation.
- c) Noncompliance with the protocol.
- d) Investigator decision to withdraw a subject from study.
- e) Subjects, who are prescribed medication for an illness arising during the study, may be terminated on the basis of an intercurrent event. This event will be noted on the appropriate CRF's.
- f) Subjects who decide to withdraw from the study for personal reasons.

D. Investigator Review

The Investigator will review all case report forms and will sign the Investigator Review Form (CRF 6) at study termination attesting to the completeness and accuracy of case report forms that pertain to their responsibilities.

VII. Statistical Analyses

Data will be analyzed using analysis of variance. Data will be analyzed according to a 3 x 3 factorial design. Factors will be treatment levels and subject. Additional terms to be included in the model provided there are sufficient degrees of freedom are side, arm site, and the side by site interaction. P-values ≤ 0.10 will be considered significant.

VIII. Ethical and Regulatory Requirements

A. Institutional Review Board (IRB) Review and Approval

Review by an IRB is required to conduct this study. A copy of the approval letter along with a list of the IRB members who acted on this protocol and a statement that the IRB is in compliance with current Good Clinical Practices (GCP) regulations will be provided to the Sponsor.

B. Subject Informed Consent

Prior to study initiation, all subjects will be informed as to the type of study, the procedures to be followed, the general nature of the products being tested, and any known or anticipated adverse reactions which might result from participation. Each subject must sign the written informed consent (Appendix A) before participating in this study. The informed consent will contain all the basic elements outlined in 21 CFR 50.25.

C. Study Monitoring

The Investigator will permit a representative of the Sponsor to make regular visits during the course of the study. During these visits, the Investigator will permit the Sponsor's Monitor to inspect all forms and corresponding study subject's records to verify adherence to the protocol. The Sponsor's Monitor will also be permitted to review and verify laboratory reports, case report forms, drug/test article supply and inventory records. Any comments/instructions made by the Sponsor's Monitor will be recorded in the Investigator's study file.

D. Protocol Revisions and Amendments

With the exception of emergency situations, no changes or deviations from this protocol will be permitted without documented approval from the Investigator and the Sponsor's Monitor.

All amendments to the final protocol will be initiated by the Sponsor. They will be consecutively numbered, describe any changes being made, and the reasons for them. All amendments will be signed and dated by the Sponsor and the Investigator, and the impact on the study noted. If the Investigator deviates from the agreed final protocol, the Sponsor's Monitor will be informed of the change as soon as possible by telephone.

E. Final Report

The Sponsor will generate a final report of clinical results.

F. Study Safety Statement

The requested testing meets the ethical requirements stipulated in the Procter & Gamble Policy for Research Involving Human Subjects. Appropriate safety testing has been completed and risk assessments justify the placement of the test products in this study at these concentrations (levels of exposure).

G. Confidentiality

The obligations of the Investigator, Hill Top Research, Inc., regarding the confidential information on the antibacterial soap and all aspects of the study will be kept confidential according to the Laboratory Service agreement between Hill Top Research, Inc. and The Procter & Gamble Company.

IX. References

1. Williamson, P. and Kligman, A.M., A new method for the quantitative investigation of cutaneous bacteria. *J. Invest. Dermatol.*, 45:6 (1965) 498-503.

X. Sponsor and Investigator Concurrence

For The Procter and Gamble Company

PREPARED BY:

Jan S. Englehart

Jan S. Englehart, B.S., ASCP, Clinical Research Associate
Clinical Research and Biometrics Department

Date: 11/18/97

APPROVED BY:

Ward L. Billhimer

Ward L. Billhimer, M.S., Senior Scientist
Clinical Research and Biometrics Department

Date: 11/18/97

Paula B. Neumann


Paula B. Neumann, Ph.D., Senior Scientist Biostatistician
Clinical Research and Biometrics Department

Date: 11/18/97

Agreed and Accepted by Hill Top Research, Inc. and the Study Investigator for
CRB-97-11-245-CD:

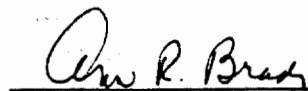
I certify that I have reviewed and approved the protocol, informed consent form, and other associated documents and agree to abide by their terms. In addition, I agree to conduct this clinical study in compliance with federal, state and local government regulations, guidelines and standards applicable to such studies including, but not limited to, those relating to Institutional Review Board (IRB), Informed Consent, and Good Clinical Practices.

I am aware that it is the responsibility of the Investigator to promptly report to the IRB all changes to the research activity and all unanticipated problems involving risk to human subjects. In addition, as Investigator, I am aware that a summary report must be submitted to the IRB when the study is completed. These guidelines are in accordance with CFR 312.66. The Sponsor will be copied on all correspondence to and from the IRB.



Gayle K. Mulberry, M.S., Principal Investigator

Date: 11-19-97



Ann R. Brady, A.S., Sub-Investigator

Date: 11-19-97

XI. Attachments

The following Appendices, Case Report Forms, and Source Documents are included as attachments to the Final Protocol:

Appendices

- A Written Informed Consent
- B Subject's Instructions Following Study Completion
- C Wash Procedure
- D Product Labels
- E List of Antibacterial/Antimicrobial Products
- F Microbiological Media

Case Report Forms

- 1 Demographics/Dermatological/Medical History Form
- 2 Inclusion/Exclusion Form
- 3 Treatment Record
- 4 Adverse Event
- 5 Physician's Action Report Form
- 6 Subject Termination and Investigator Review Form

Source Documents

- 1 Shipping and Receiving of Study Material
- 2 Microbiology Worksheet - Enumeration of Organisms

Appendix A

**HT# 97-5425-11
CRB-97-11-245-CD**

WRITTEN INFORMED CONSENT

To be provided by the clinical site.

Appendix B

**HT# 97-5425-11
CRB-97-11-245-CD**

SUBJECT'S INSTRUCTIONS FOLLOWING STUDY COMPLETION

You have just completed participation in a clinical study, "Residual Effectiveness Screening Test". During this study, two (2) test sites on each of your forearms were inoculated with *Escherichia coli* bacteria. Although we do not expect you have any adverse experience as a result of participation in this study, there is a remote possibility that an infection may develop on your forearms within the next 48 - 72 hours.

To determine whether you have developed an infection from the test bacteria, we would like you to examine your arms during the next 48 - 72 hour period. If you notice the appearance of any pimples, blisters, or raised red itching bumps surrounded by redness and/or swelling, please contact Gayle Mulberry or Ann Brady at (513) 831-3114 during normal business hours (8:15 am - 5 pm) or at (513) 831-3354 after hours.

Thank you for your cooperation.

Appendix C
HT# 97-5425-11
CRB-97-11-245-CD

WASH PROCEDURE

Water temperature should be maintained at 95 -100° F.

The temperature should be checked and recorded before each wash.

Water flow should be 4 L/minute.

Time of each wash should be recorded.

A technician will wash each subject's arm.

The technician will wear gloves for this procedure, changing after each treatment area wash.

Wipe the template with 70% isopropyl alcohol after use.

Begin with the subject's right arm:

- 1. Using the template, mark two (2) 10 x 5 cm treatment areas (an upper and lower) on the subject's forearm.**
- 2. The subject should wet the upper treatment site of their forearm under the running water.**
- 3. Dispense 0.5 mL of the appropriate test product, from a 1 cc disposable syringe, onto the upper treatment site area.**
- 4. The technician should wet their gloved hand under the running water.**
- 5. The technician should carefully lather the test product with two (2) fingers in an up-and-down motion within the upper treatment site for forty-five (45) seconds.**
- 6. The subject should rinse the upper treatment site avoiding crossover to the lower treatment site under the running water. Rinse for fifteen (15) seconds. Do not rub!**
- 7. Repeat steps 1 to 6 for the lower treatment site.**
- 8. Pat subjects' forearms dry using a paper towel. Do not rub!**
- 9. Repeat steps 1 to 8 on the left forearm.**

Appendix D

HT# 97-5425-11
CRB-97-11-245-CD

PRODUCT LABELS

**ANTIBACTERIAL
LIQUID SOAP PRODUCT
Product Code A**

Study# CRB-97-11-245-CD

HT#97-5425-11

Net Contents: 100 g

Distributed by:

Hill Top Research
Main and Mill Streets
Miamiville, Ohio 45147
(513) 831-3354

May Contain: Triclosan (TCS)

Exp. Date: 1/1/98

Use as directed for washing
arms only.

**ANTIBACTERIAL
LIQUID SOAP PRODUCT
Product Code B**

Study# CRB-97-11-245-CD

HT#97-5425-11

Net Contents: 100 g

Distributed by:

Hill Top Research
Main and Mill Streets
Miamiville, Ohio 45147
(513) 831-3354

May Contain: Triclosan (TCS)

Exp. Date: 1/1/98

Use as directed for washing
arms only.

**ANTIBACTERIAL
LIQUID SOAP PRODUCT
Product Code C**

Study# CRB-97-11-245-CD

HT#97-5425-11

Net Contents: 100 g

Distributed by:

Hill Top Research
Main and Mill Streets
Miamiville, Ohio 45147
(513) 831-3354

May Contain: Triclosan (TCS)

Exp. Date: 1/1/98

Use as directed for washing
and arms only.

**ANTIBACTERIAL
LIQUID SOAP PRODUCT
Product Code D**

Study# CRB-97-11-245-CD

HT#97-5425-11

Net Contents: 100 g

Distributed by:

Hill Top Research
Main and Mill Streets
Miamiville, Ohio 45147
(513) 831-3354

May Contain: Triclosan (TCS)

Exp. Date: 1/1/98

Use as directed for washing
and arms only.

Appendix D

**HT# 97-5425-11
CRB-97-11-245-CD**

PRODUCT LABELS

**ANTIBACTERIAL
LIQUID SOAP PRODUCT
Product Code E**

**Study# CRB-97-11-245-CD
HT#97-5425-11**

Net Contents: 100 g

**Distributed by:
Hill Top Research
Main and Mill Streets
Miamiville, Ohio 45147
(513) 831-3354**

**May Contain: Triclosan (TCS)
Exp. Date: 1/1/98**

**Use as directed for washing
arms only.**

**ANTIBACTERIAL
LIQUID SOAP PRODUCT
Product Code F**

**Study# CRB-97-11-245-CD
HT#97-5425-11**

Net Contents: 100 g

**Distributed by:
Hill Top Research
Main and Mill Streets
Miamiville, Ohio 45147
(513) 831-3354**

**May Contain: Triclosan (TCS)
Exp. Date: 1/1/98**

**Use as directed for washing
arms only.**

**ANTIBACTERIAL
LIQUID SOAP PRODUCT
Product Code G**

**Study# CRB-97-11-245-CD
HT#97-5425-11**

Net Contents: 100 g

**Distributed by:
Hill Top Research
Main and Mill Streets
Miamiville, Ohio 45147
(513) 831-3354**

**May Contain: Triclosan (TCS)
Exp. Date: 1/1/98**

**Use as directed for washing
arms only.**

**ANTIBACTERIAL
LIQUID SOAP PRODUCT
Product Code H**

**Study# CRB-97-11-245-CD
HT#97-5425-11**

Net Contents: 100 g

**Distributed by:
Hill Top Research
Main and Mill Streets
Miamiville, Ohio 45147
(513) 831-3354**

**May Contain: Triclosan (TCS)
Exp. Date: 1/1/98**

**Use as directed for washing
arms only.**

Appendix D

**HT# 97-5425-11
CRB-97-11-245-CD**

PRODUCT LABELS

**ANTIBACTERIAL
LIQUID SOAP PRODUCT
Product Code I**

Study# CRB-97-11-245-CD

HT#97-5425-11

Net Contents: 100 g

Distributed by:

Hill Top Research

Main and Mill Streets

Miamiville, Ohio 45147

(513) 831-3354

May Contain: Triclosan (TCS)

Exp. Date: 1/1/98

**Use as directed for washing
and arms only.**

**ANTIBACTERIAL
LIQUID SOAP PRODUCT
Product Code J**

Study# CRB-97-11-245-CD

HT#97-5425-11

Net Contents: 100 g

Distributed by:

Hill Top Research

Main and Mill Streets

Miamiville, Ohio 45147

(513) 831-3354

May Contain: Triclosan (TCS)

Exp. Date: 1/1/98

**Use as directed for washing
and arms only.**

Appendix E
HT# 97-5425-11
CRB-97-11-245-CD

LIST OF ANTIBACTERIAL / ANTIMICROBIAL PRODUCTS

Medicated Acne Cleansers

Benzac W Wash 5
Desuam-X 5 Wash
Benzac W Wash 10
Desquam-X 10m Wash
Fostex 10% BPO Wash
Oxy 10 Wash
Propa P.H. Liquid Acne Soap
PanOxyl 5
Fostex 10% BPO
PanOxyl 10
Clearasil Antibacterial Soap
Sastid Plain Therapeutic Shampoo and Acne Wash
Oxy Clean Soap
Fostex Medicated Cleansing Bar
Salicylic Acid and Sulfur Soap
Sulfur Soap

Antidandruff Shampoos

Head and Shoulders (all formulas)
Selsun Blue (all formulas)
Pert Plus for Dandruff
Suave for dandruff
Neutrogena T-gel
Neutrogena T-sal
Scalpacin
Tegrin
Any antidandruff shampoo

Anti-bacterial Soaps

Safeguard bar and liquid
Lever 2000 bar and liquid
Irish Spring bar
Dial bar and liquid
Softsoap Antibacterial Soap

Antibiotic Ointments and Creams

Bacitracin
Polysporin
J & J First Aid Cream
Neomycin

Anti-bacterial Dishwashing Liquids

Dawn
Joy
Palmolive
Dial

Appendix F
HT# 97-5425-11
CRB-97-11-245-CD

MICROBIOLOGICAL MEDIA

0.075M Phosphate Buffer Solution

Weigh 0.4 grams of KH_2PO_4 , 10.1 grams of Na_2HPO_4 , 1.0 gram Triton X, 15.0 grams of polysorbate (Tween) 80, and 10.0 grams of Lecithin. Dissolve in 1 liter of distilled or deionized water. Adjust to pH 7.9 with 0.1N NaOH. Dispense buffer in 100 mL quantities in bottles. Loosely cap bottles and sterilize in the autoclave at 121°C. Prepared buffer is checked for sterility and stored at 15 - 30°C for upto 30 days.

Trypticase Soy Broth (TSB)

Dissolve 30 grams in 1 liter of distilled or deionized water. If necessary, warm slightly to dissolve completely. Dispense broth in 9 mL quantities in sterile tubes. Sterilize at 121°C. Check for sterility. Prepared tubes are stored at 15 - 30°C and used within 30 days.

Trypticase Soy Agar with Polysorbate (Tween) 80

Suspend 40 grams in 1 liter of distilled or deionized water in a heat resistant flask. Heat to boiling with gentle mixing to dissolve completely. Add 15 grams of polysorbate (Tween) 80 and gently mix to dissolve completely. Loosely cap flask and sterilize in the autoclave at 121°C. Cool to 45 - 50°C in a water bath. Pour in sterile 15 x 100 mm Petri dishes. Allow to cool and solidify on a level flat surface. Check for sterility. Prepared plates are stored at 2 - 8°C and used within 30 days.

Estimated Plate Count Procedure

Do not record counts on crowded plates from the highest dilution as too numerous to count (TNTC). If the number of colonies per plate exceeds 250, count colonies in those portions of the plate that are representative of colony distribution and calculate the Estimated Standard Plate Count (ESPC) from these counts. The ESPC will be determined utilizing the grid embossed area on the lighted surface of the colony counter. Each large square on the grid is 1 cm². If there are fewer than 10 colonies per square centimeter, count colonies in 12 squares, selecting, if representative, six consecutive squares horizontally across the plate and six consecutive squares at right angles, being careful not to count a square more than once. When there are more than 10 colonies per square centimeter, count colonies in four such representative portions. In both instances, multiply the average found per square centimeter by the area of the plate used to determine the estimated number of colonies per plate.

If the total number of CFU's have been estimated according to the procedure described above, ESPC (Estimated Standard Plate Count) should be recorded following the value.

Note: If the highest dilution plated contains >250 CFU's and a count ≤300 CFU's has been previously determined, that value may be reported. It will not be necessary to estimate the total CFU's on a plate containing >250 CFU's using the above procedure. Plates containing the highest dilution of test specimen plated and the CFU counts are greater than 300, then the above procedure should be used to determine the total CFU count.

Case Report Form 1
DEMOGRAPHICS/DERMATOLOGICAL/MEDICAL HISTORY FORM

Study #	Hill Top Research, Inc.	Visit Code	Date	Subject Initials	Subject Screen #
97-5425-11	Gayle K. Mulberry	Subject Qualification	____/____/____ mm dd yy	____/____/____ F M L	Permanent #:

Gender: <input type="checkbox"/> Male ⁽¹⁾ <input type="checkbox"/> Female ⁽²⁾	Age: _____ Years
---	------------------

Does the subject have any of the following at the treatment sites?

I. DERMATOLOGIC DISORDER	No	Yes	Don't Know
1. Psoriasis ?			
2. Eczema ?			
3. Skin Cancer ?			
4. Skin Allergies ? Please specify:			
5. Hives ?			

Does the Subject have any of the following (present and past)?

II. OTHER MEDICAL INFORMATION	No	Yes	Don't Know
1. Allergies ? Please specify.			
2. Hepatitis ?			
3. Heart and Vascular Disease?			
4. Liver Disease ?			
5. Kidney Disease ?			
6. Tuberculosis ?			
7. Diabetes ? Controlled? Diet [] Oral [] Insulin []			
8. Cancer ?			
9. Auto-immune disease (Lupus erythematosus, thyroiditis, AIDS, etc.) ?			
10. Organ transplant ?			
11. Any other condition not listed ? Please specify			

Is the subject taking any medication? If yes, please specify below:

III. MEDICATION	No	Yes	Don't Know
1. Antibiotics, oral or systemic ?			
2. Cortisone, Steroids, ACTH, Anti-reaction Drugs ?			
3. Heart Medication ?			
4. Insulin ?			
5. Other ?			

Comments:

Based on the above medical history, the subject is: ☐ Qualified or ☐ Not qualified for the study.

Interviewer's Signature:	Date: ____/____/____ mm dd yy
--------------------------	----------------------------------

**Case Report Form 2
INCLUSION / EXCLUSION FORM**

Study #	Hill Top Research, Inc.	Visit Code	Date	Subject Initials	Subject Screen #
97-5425-11	Gayle K. Mulberry	Subject Qualification	____/____/____ mm dd yy	____/____/____ F M L	Permanent #:

INCLUSION CRITERIA

Check one		
YES	NO	Subject:
		1. Is 18 to 65 years ?
		2. Has signed informed consent ?
		3. Is healthy as evidenced by responses on CRF 1 ?
		4. Has forearms that are free of dermatoses, cuts, lesions, and other skin disorders ?
		5. Is willing to comply with all study protocol requirements ?

EXCLUSION CRITERIA

Check one			
YES	NO	N/A	Subject:
			1. Is currently participating in another clinical study at this or any other facility ?
			2. Has participated in any type of hand or arm wash study within the past 14 days ?
			3. Has cuts, lesions, or other skin disorders on the volar surface of either forearm ?
			4. Has soap, detergent, and/or perfume allergies ?
			5. Has eczema or psoriasis on their arm(s) ?
			6. Has taken systemic antibiotics or used topical antibiotics for any reason in the 3 weeks prior to the start of the test period ?
			7. Are currently using antibacterial/antimicrobial soaps (liquids and/or bars), medicated lotions and creams, and/or anti-dandruff shampoos ?
Female	Female	Male	8. Is currently pregnant ? <input type="checkbox"/> Yes <input type="checkbox"/> No Of child-bearing potential: <input type="checkbox"/> Yes <input type="checkbox"/> No <input type="checkbox"/> Surgically Sterile <input type="checkbox"/> Post-menopausal If of child bearing potential - β -HCG Test Results: <input type="checkbox"/> negative <input type="checkbox"/> positive
Female	Female	Male	9. Is currently lactating?
			10. Has been medically diagnosed as having a medical condition such as: diabetes, hepatitis, an organ transplant, or AIDS (or HIV positive) ?
			11. Has another medical condition which in the opinion of the Investigator would preclude participation ?

BASED UPON DERMATOLOGIC EVALUATION AND THE INFORMATION CONTAINED IN CASE REPORT FORMS 1 AND 2, THE SUBJECT IS:

☐ **Qualified** ☐ **Not Qualified** for participation in this study.

Reasons for disqualification: _____

Investigator's Signature: _____	Date: ____/____/____ mm dd yy
--	---

**Case Report Form 3
TREATMENT RECORD**

Study #	Hill Top Research, Inc.	Visit Code	Subject Initials	Permanent #:
97-5425-11	Gayle K. Mulberry	Test Period	____/____/____ F M L	

WASH RECORD						
Wash	Right Arm Test Sites		Left Arm Test Sites		Water Temp.	Initials / Date
	Upper (1)	Lower (2)	Upper (3)	Lower (4)		
Product Code						
1	am / pm	am / pm	am / pm	am / pm		/

INOCULATION, OCCLUSION, AND HARVESTING					
Right Arm	Site	Time Period	Inoculation and Occlusion Time	Harvesting Time	Initials / Date
	<i>upper (1)</i>		am / pm	am / pm	/
	<i>lower (2)</i>		am / pm	am / pm	/
Left Arm	Site	Time Period	Inoculation and Occlusion Time	Harvesting Time	Initials / Date
	<i>upper (3)</i>		am / pm	am / pm	/
	<i>lower (4)</i>		am / pm	am / pm	/

Microbiologist's Initials:	Investigator's Signature:	Date: ____/____/____ mm dd yy
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Case Report Form 4

ADVERSE EVENT

Study #	Hill Top Research, Inc.	Date	Subject Initials	Permanent #:
97-5425-11	Gayle K. Mulberry	____/____/____ mm dd yy	____/____/____ F M L	

Was reaction related to treatment? ☐ Not related ☐ Possibly related ☐ Definitely related ☐ Other (explain)

Did subject take any medication during the study period? ☐ YES ☐ NO If yes, complete section below.

Date of Onset: _____ Date Reported: _____ Date Resolved: _____

Describe event: _____

Action Taken: ☐ None ☐ Continued on study ☐ Withdrawn from the study ☐ Consulted physician

☐ Medication taken (Complete below) ☐ Hospitalized ☐ Other (explain)

Additional Comments:

FOLLOW - UP ACTION TAKEN

Date	Action Taken	Comments	Initials

CONCOMITANT MEDICATION TAKEN

Medication (Oral or Systemic)	Total Daily Dose	Start Date mm / dd / yy	Stop Date mm / dd / yy	Indication (Reason for Taking)
		/ /	/ /	
		/ /	/ /	
		/ /	/ /	

Investigator's Signature:

Recorded by:

Date

____/____/____
mm dd yy

Case Report Form 5

PHYSICIAN'S ACTION REPORTING FORM

Study #	Hill Top Research, Inc.	Date	Subject Initials	Permanent #:
97-5425-11	Gayle K. Mulberry	____/____/____ mm dd yy	____/____/____ F M L	

Date(s) of office visit(s): _____

Pertinent Medical History: (e.g., causes of similar reactions, known allergies,
potential involvement of current medications or medical conditions)

Test Product Exposure:

Use Began On: _____ Used Ended on: _____ Number of Uses: _____

Date Date

Clinical Observations: (Include date of onset and descriptions/severity/locations, etc.)

Impression: _____

Treatment: _____

Follow Up: _____

Date Resolved: _____

Is condition related to use of the test products?

☐ Probably related* ☐ Not Related* ☐ Unknown

Reasons: _____

Physician's Signature_____
Date

Case Report Form 6

SUBJECT TERMINATION AND INVESTIGATOR REVIEW FORM

Study #	Hill Top Research, Inc.	Visit Code	Subject Initials	Permanent #:
97-5425-11	Gayle K. Mulberry	End of Study	____/____/____ F M L	

Date Subject Entered the Study: ____/____/____ mm dd yy	Date Subject Withdrew / Completed the Study: ____/____/____ mm dd yy
---	--

Did subject drop out of study prior to completion? ☐ YES ☐ NO If yes, complete below:

Check reason for subject premature termination:

- ☐ 1 = Adverse Event (Documented on CRF 4)
- ☐ 2 = Intercurrent Event
- ☐ 3 = Lack of Compliance with Protocol Specify: _____
- ☐ 4 = Personal reasons (family problems, lack of transportation, etc.)
- ☐ 5 = No show, Lost to Follow-up
- ☐ 6 = Other Specify: _____

Comments: _____

**MY SIGNATURE ON THIS PAGE CERTIFIES
THAT I HAVE REVIEWED ALL OF THE DATA
AND STATEMENTS SUBMITTED FOR THIS
SUBJECT AND FIND THEM ACCURATE AND
COMPLETE TO THE BEST OF MY KNOWLEDGE.**

Investigator's Signature	Date ____/____/____ mm dd yy
--------------------------	------------------------------------

SOURCE DOCUMENT 1
CRB-97-11-245-CD
(HT# 97-5425-11)

SHIPPING & RECEIVING OF STUDY MATERIAL

MATERIALS SHIPPED		MATERIALS RECEIVED	MATERIALS RETURNED	MATERIALS RECEIVED
Date Shipped: _____ Time: _____		Date Received: _____	Date: _____ Time: _____	Date Received: _____
Test Site: _____		Time: _____	Test Site: _____	Time: _____
Method of Transport: _____		Test Site: _____	Method of Transport: _____	Test Site: _____
LISTING OF MATERIALS SHIPPED		MATERIALS RECEIVED	MATERIALS RETURNED	MATERIALS RETURNED
# of ITEMS	ITEM DESCRIPTION * * Include Batch no. in item description	4= OK D = LOST/DAMAGED NR = NOT RECEIVED	Indicate if items are returned or not returned	4= OK D = LOST/DAMAGED NR = NOT RECEIVED

Shipping Technician: _____ Date: _____	Receiving Technician: _____ Date: _____	Returning Technician: _____ Date: _____	Receiving Technician: _____ Date: _____
---	--	--	--

SOURCE DOCUMENT 2

HT# 97-5425-11

CRB-97-11-245-CD

MICROBIOLOGY WORKSHEET - ENUMERATION OF ORGANISMS

CFU COUNTS (TOTAL # ORGANISMS / mL)									
Date Plated:			Date Counted:				Inoculum Count:		
Subject No.	Test Site	Right Arm				Left Arm			
		1 (upper)		2 (lower)		3 (upper)		4 (lower)	
		Plate 1	Plate 2	Plate 1	Plate 2	Plate 1	Plate 2	Plate 1	Plate 2
	10 ⁻¹								
	10 ⁻²								
	10 ⁻³								
	10 ⁻⁴								
	10 ⁻⁵								
Subject No.	Test Site	Right Arm				Left Arm			
		1 (upper)		2 (lower)		3 (upper)		4 (lower)	
		Plate 1	Plate 2	Plate 1	Plate 2	Plate 1	Plate 2	Plate 1	Plate 2
	10 ⁻¹								
	10 ⁻²								
	10 ⁻³								
	10 ⁻⁴								
	10 ⁻⁵								
Subject No.	Test Site	Right Arm				Left Arm			
		1 (upper)		2 (lower)		3 (upper)		4 (lower)	
		Plate 1	Plate 2	Plate 1	Plate 2	Plate 1	Plate 2	Plate 1	Plate 2
	10 ⁻¹								
	10 ⁻²								
	10 ⁻³								
	10 ⁻⁴								
	10 ⁻⁵								
Subject No.	Test Site	Right Arm				Left Arm			
		1 (upper)		2 (lower)		3 (upper)		4 (lower)	
		Plate 1	Plate 2	Plate 1	Plate 2	Plate 1	Plate 2	Plate 1	Plate 2
	10 ⁻¹								
	10 ⁻²								
	10 ⁻³								
	10 ⁻⁴								
	10 ⁻⁵								
Laboratory Supervisor			Microbiologist				Date		

PROTOCOL AMENDMENT Issue I 11/24/97

Residual Effectiveness Screening Test of Antibacterial Liquid Products against *E. coli*

Study Number: CRB-97-11-245-CD (HT 97-5425-11)

Purpose of the
Amendment: Changes to the Study Protocol

CHANGE #1 Clarification of the Title of the Protocol

The title of the protocol will be modified to read as follows:

"Residual Effectiveness Screening Test of Liquid Products against *E. coli*"

The word "antibacterial" has been removed since all of the test products do not contain the antibacterial active ingredient.

CHANGE #2 Clarification of the Objective of the Protocol

The objective of the protocol will be modified to read as follows:

"The objective of this study is to evaluate the residual effectiveness of ten (10) liquid soap products against potentially pathogenic bacteria (*Escherichia coli*, ATCC 11229) under simulated skin conditions which are considered optimal for bacterial growth, proliferation, and possible infection."

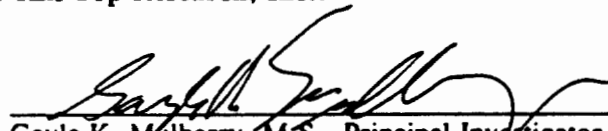
The words "containing an antibacterial active" have been removed since all of the test products do not contain the antibacterial active ingredient.

For The Procter and Gamble Company:


J. S. Englehart, B.S., Clinical Research Associate

Date: 11/24/97

For Hill Top Research, Inc.:


Gayle K. Mulberry, M.S., Principal Investigator

Date: 11/24/97


Ann R. Brady, A.S., Sub-Investigator

Date: 11-24-97

FINAL REPORT OF CLINICAL TEST RESULTS

From: P. B. Neumann, J. S. Englehart

Date: July 10, 1998

To: Study File for CRB-97-12-262-CD

Retention Limit: Until Superseded

Released: WLB 7/10/98

Subject: Residual Effectiveness Screening Test Results on B-22M Liquid Soap Formulations with Varying Levels of Salicylic Acid and Triclosan (TCS) and Liquid Ivory® against *E. coli* under Occluded and Unoccluded Conditions
Hill Top Research, Inc., CRB-97-12-262-CD, HT 97-5461-11

Summary:

The results of this clinical study show that B-22M liquid soap formulation with 1.0% salicylic acid, code G, and B-22M liquid soap formulation with 1.0% salicylic acid and 1.0% TCS, code I, were significantly more effective than B-22M (no salicylic acid or TCS, code A) and Liquid Ivory, code J, at lowering the levels of *Escherichia coli* inoculated on the skin (p-value <0.10) under both occluded and unoccluded conditions. Treatment and occlusion status were highly significant factors, their interaction was not.

The results are summarized in the attached tables.

Objective:

The objective of this study was to evaluate the residual effectiveness of four (4) liquid soap products against potentially pathogenic bacteria (*Escherichia coli*, ATCC 11229) under simulated skin conditions which are considered optimal for bacterial growth, proliferation, and possible infection.

Test Products:

Code	Test Product	Active Ingredient	TSIN
A	B-22M	none	BI0060-108
G	B-22M, 1.0% salicylic acid	none	BI0060-092
I	B-22M, 1.0% salicylic acid	1.0% TCS	BI0060-096
J	Liquid Ivory Soap	none	BI0060-126

Study Design:

This study was conducted at Hill Top Research, Inc. in Miamiville, Ohio on December 2, 1997. This was a randomized, split forearm wash study to evaluate the residual antibacterial effectiveness of four (4) liquid products. Twenty (20) male and female subjects, ages 18 through 65 years old, who did not regularly use antibacterial soap, medicated lotion or cream, and/or antidandruff shampoo were enrolled to evaluate the products under both occluded and unoccluded conditions. For the test procedure, each of the subject's forearms was divided into an upper and lower treatment area, for a total of four (4) treatment areas per subject. Each treatment area was washed one (1) time for forty-five (45) seconds. Within five (5) minutes after the wash with the test products was completed, a 3.0 cm circular test site was marked-off in each area. Each circular site was then inoculated with 10 µL of a 24 hour broth culture of *E. coli* grown in Trypticase Soy Broth (TSB). According to a randomization, half of the test sites were occluded with a 24 mm Hill Top Chamber® that was taped to the skin with Durapore® tape. The other half of the test sites remained

Study Design: (continued)

unoccluded. Ten (10) minutes after inoculation, the surviving organisms were harvested from each test site using the Williamson-Kligman scrub technique. The specimens from each site were plated on Trypticase Soy Agar with polysorbate (Tween) 80 and incubated for 18 - 24 hours at $35 \pm 2^\circ\text{C}$. The colony forming units (CFU's) of *E. coli* were counted at the end of the incubation period to determine the number of surviving organisms at each of the treated sites.

Data Analysis:

The surviving colony forming units (CFU's) of bacteria for each subject were enumerated. The numbers of bacteria were converted to base 10 logarithms. The \log_{10} CFU counts were compared using analysis of variance techniques according to a 4 x 2 factorial design with factors of treatment, occlusion and subject, adjusting for subject to subject variability, side (right vs. left) variability, site (upper vs. lower) variability, and site to side variability, to estimate which of the test products had the greatest activity. P-values ≤ 0.10 were considered statistically significant.

Subject Accountability:

Twenty (20) subjects were enrolled and completed this study.

Adverse Events:

None reported.

Submitted by:

Paula B. Neumann
Paula B. Neumann

Jan S. Englehart
Jan S. Englehart

Treatments:

A = B22M
 G = B22M, 1% Salicylic Acid
 I = B22M, 1% Salicylic Acid, 1% TCS
 J = Liquid Ivory Soap

Conclusions:

Treatment and occlusion status were highly significant factors. The interaction between these terms was not significant.

Significantly higher lsmean organism counts (*E. coli*) were detected with occlusion (p-value = 0.0003).

Liquid Ivory Soap sites had higher organism counts than either B22M with 1% salicylic acid or B22M with 1% salicylic acid & 1% TCS at p-values < 0.10.

B22M sites had higher organism counts than B22M with both salicylic acid and TCS (p-value = 0.04).

Occlude	Treatment	N	Mean	Std. Dev.
No	A	10	4.8	0.320
No	G	9	4.5	0.484
No	I	9	4.2	0.209
No	J	10	5.0	0.159
Yes	A	10	5.1	0.630
Yes	G	9	4.9	0.569
Yes	I	9	4.7	0.795
Yes	J	10	5.4	0.594

Std.

Occlude	LSMean	Error	p-value = 0.0003
No	4.64	0.074	
Yes	5.06	0.074	

Treatment	LSMean	Std. Error	Pairwise p-values			
			A	G	I	J
A	4.9	0.124	A	0.4383	0.0379	0.2443
G	4.8	0.134	G	0.4383	0.1138	0.1049
I	4.5	0.134	I	0.0379	0.1138	0.0006
J	5.1	0.126	J	0.2443	0.1049	0.0006
Overall p-value=0.0072						

Std. LSMean

Treatment	Occlude	LSMean	Error	#	
A	No	4.8	0.161	1	
A	Yes	5.1	0.161	2	
G	No	4.6	0.173	3	
G	Yes	5.0	0.173	4	Overall p-value=0.8832
I	No	4.2	0.172	5	
I	Yes	4.8	0.172	6	
J	No	5.0	0.163	7	
J	Yes	5.4	0.163	8	

Pairwise p-values (Note: These are not valid as the overall p-value is not significant.)

i/j	1	2	3	4	5	6	7	8
1		0.1239	0.4729	0.3715	0.0333	0.9620	0.4449	0.0178
2	0.1239		0.0408	0.6374	0.0012	0.2009	0.5370	0.2961
3	0.4729	0.0408		0.0886	0.1139	0.5267	0.1891	0.0068
4	0.3715	0.6374	0.0886		0.0028	0.3683	0.9015	0.1794
5	0.0333	0.0012	0.1139	0.0028		0.0138	0.0027	0.0001
6	0.9620	0.2009	0.5267	0.3683	0.0138		0.4187	0.0175
7	0.4449	0.5370	0.1891	0.9015	0.0027	0.4187		0.0664
8	0.0178	0.2961	0.0068	0.1794	0.0001	0.0175	0.0664	

**RESIDUAL EFFECTIVENESS SCREENING TEST OF
ANTIBACTERIAL LIQUID PRODUCTS AGAINST *E. COLI* UNDER OCCLUDED
AND UNOCCLUDED CONDITIONS**

Study Number: CRB-97-12-262-CD

Study Identification: HT# 97-5461-11

Principal Investigator: Gayle K. Mulberry, M.S.

Sub-Investigator: Ann R. Brady, A.S.

Clinical Test Site: Hill Top Research, Inc.
Main and Mill Streets
Miamiaville, Ohio 45147

Sponsor: The Procter & Gamble Company
Clinical Research and Biometrics Division
Personal Cleansing Sector
11511 Reed Hartman Highway
Cincinnati, Ohio 45241

Sponsor Representative: Ward L. Billhimer, M.S.

Sponsor Statistician: Paula B. Neumann, Ph.D.

Sponsor Toxicologists: Paul F. Sterchele, Ph.D.
J. David Innis, Ph.D.

Clinical Research Associates: Jan S. Englehart, B.S., ASCP

Start Date: December 2, 1997

Confidentiality: The obligations of the Investigator, regarding the confidential information on the antibacterial soap and all aspects of the study will be kept confidential according to the agreement with The Procter & Gamble Company.

I. Study Objective

The objective of this study is to evaluate the residual effectiveness of four (4) liquid soap products containing an antibacterial active against potentially pathogenic bacteria (*Escherichia coli*, ATCC 11229) under simulated skin conditions which are considered optimal for bacterial growth, proliferation, and possible infection.

II. Study Summary

This is a randomized blinded clinical study that will evaluate the residual effectiveness of four (4) liquid soap products using a split forearm test design. It will consist of a one (1) day test period. Twenty (20) male and female subjects, age 18 to 65 years, who do not regularly use antibacterial/antimicrobial soaps, medicated lotions and creams, and/or anti-dandruff shampoos will be enrolled into the study. During this study, twenty (20) subjects will be used to evaluate four (4) test products under both occluded and unoccluded conditions.

On the day of the test, subjects will report to the clinical test facility. Each of the subjects' forearms will be divided into an upper and lower treatment area for a total of four (4) treatment areas. Subjects will have each of their forearms washed by a laboratory technician with the test products according to a randomization.

Following treatment with the test products, a test site will be marked off in the center of each treatment area on the forearms. Each of the four (4) test sites will be inoculated with a known amount of *Escherichia coli* (ATCC 11229) grown in Trypticase Soy Broth (TSB). According to a randomization, half of the test sites will then be occluded with a Hill Top Chamber®. The other half of the test sites will remain unoccluded. After ten (10) minutes, the bacteria on the skin will be harvested using a scrub technique ⁽¹⁾. Each sample of harvested bacteria will be diluted, plated, and incubated. Following incubation, the number of surviving colony forming units (CFU's) for each site will be determined.

III. Study Population

Subjects will be screened for their eligibility to participate based upon information provided in the Demographics/Dermatological/Medical History Form [Case Report Form (CRF) 1]. Only subjects meeting the inclusion/exclusion criteria, outlined in CRF 2, will be allowed to participate in the study. If a subject is admitted to this study in apparent violation of any of the above criteria, the reason(s) for admission will be noted by the Investigator or his designee.

A. Subject Inclusion Criteria

Subjects are eligible for enrollment if they:

1. Are a male or female, age 18 to 65 years;
2. Have signed the Informed Consent (Appendix A);
3. Are in good health, as evidenced by response to the Demographics/ Dermatological/Medical History Form (CRF 1);
4. Have forearms that are free of dermatoses, cuts, lesions, and other skin disorders; and
5. Are willing to comply with all study protocol requirements.

B. Subject Exclusion Criteria

A subject cannot be enrolled in the study if they:

1. Are currently participating in another clinical study at this or any other facility;
2. Have participated in any type of hand or arm wash study within the past 14 days;
3. Have cuts, scratches, or a rash on the volar surface of either forearm;
4. Have soap, detergent, and/or perfume allergies;
5. Have eczema or psoriasis on their arm(s);
6. Have taken systemic antibiotics or used topical antibiotics for any reason in the three (3) weeks prior to the start of the study;
7. Are currently using antibacterial/antimicrobial soaps (liquids and/or bars), medicated lotions and creams, and/or anti-dandruff shampoos;
8. Are currently pregnant;
9. Are currently lactating;
10. Have been medically diagnosed as having a medical condition which would preclude participation such as: diabetes, hepatitis, an organ transplant, or AIDS (or HIV positive); and/or
11. Have any other medical condition, which in the opinion of the Investigator would preclude participation.

IV. Study Design and Procedures

A. Randomization

The Sponsor will generate the study randomization for the assignment of treatment products. In addition, a randomization will be generated to indicate which test sites will be occluded and which sites will remain unoccluded. Subjects will be assigned to a treatment number which will become their permanent subject identification number. All subjects and site personnel, including the Investigator, will remain blinded to product identities.

B. Study Schedule

On the day of the test, prospective subjects will visit the test facility to complete a written informed consent, (Appendix A), the Demographics/Dermatological/ Medical History Form (CRF 1), and the Inclusion/Exclusion Form (CRF 2). Subjects who meet the study criteria, will be randomly assigned to a treatment regimen. Two (2) treatment areas, upper and lower, will be marked off on each arm. A lab technician will wash each of the treatment areas with one (1) of the test products according to the procedure described in Appendix C. After the wash with the test products is completed, a test site will be marked-off in the center of each treatment area on the subjects' forearms. These sites will be inoculated, occluded, and harvested according to the procedures described below.

To ensure that any delayed adverse events, primarily skin infections, are reported to the study Investigator, all test subjects will be given a copy of Subjects' Instructions Following Study Completion (Appendix B) before leaving the clinical test facility after they have completed the study. This sheet will instruct the subjects to examine their forearms 48 - 72 hours after completion of the study for the presence of pimples, blisters, or raised red itching bumps surrounded by erythema and/or

edema that may be indicative of a skin infection. Subjects, who notice any of these lesions, will be instructed to call the clinical test site.

C. Wash with the Test Products

Two (2) 10 x 5 cm treatment areas (upper and lower) will be marked off on each subject's forearms using a template. Each of the four (4) treatment areas on the subjects' forearms will be washed by a laboratory technician according to the procedure outlined in Wash Procedure, Appendix C. Each treatment area will be washed one (1) time with the appropriate test product. A record of the subject washes will be kept on CRF 3. In general, the following should be noted: water temperature should be closely monitored and maintained at 95-100°F. The water temperature should be recorded. Wash time should be recorded at the start of washing.

D. Microbial Inoculation

Within five (5) minutes after the wash with the test products is completed, one (1) circular test site will be marked-off in each treatment area of the subject's forearms. These circular sites will be spaced in the center of each treatment area of the forearm. They will be made by pressing a 3.0 cm diameter glass cylinder, inked with a stamp pad, against the skin.

All test sites on both arms will be inoculated with *E. coli* (ATCC 11229) that has been grown in Trypticase Soy Broth (TSB). To determine the actual number of CFU/mL at the time of inoculation, the broth culture to be used for inoculation will be plated.

Using an Eppendorf® pipette, the skin area delineated by the cylinder will be inoculated with 10 µL of the bacterial culture to obtain 10^6 to 10^7 colony forming units (CFU's) of *E. coli*. A sterile, disposable, inoculating loop will be used to evenly spread the inoculum within the center of the test site while remaining 4 to 5 mm from the marked edge. Inoculation of each site will be documented on CRF 3.

E. Occlusion of the Test Sites

According to a randomization, half of the inoculated test sites will be immediately occluded by covering with a small plastic bowl (25 mm Hill Top Chamber® with pad removed) that will be secured to the skin with an adhesive dressing (Durapore®, 3M). The time of occlusion will be recorded on CRF 3. *The remaining half will not be occluded.* Time following inoculation should be recorded on CRF 3.

Following inoculation, all subjects will be instructed to rest their arms on a table, with the volar side of their forearms in an upward position, for approximately ten (10) minutes.

F. Harvesting of the Surviving *E. coli* Organisms

All inoculated sites will be harvested for surviving organisms at approximately ten (10) minutes after inoculation. The time of harvesting will be recorded on CRF 3.

The following procedure will be used for harvesting:

1. A hollow glass cylinder 2.2 cm in diameter will be positioned in the middle area of the test site avoiding contact with the ink-stamped edge.
2. 1.0 mL of phosphate-buffered 0.1% Triton X-100 detergent (pH 7.9), with suitable neutralizers, will be pipetted into the cylinder.

3. The skin inside the cylinder will be massaged for 60 seconds with a Teflon policeman.
4. The fluid will be removed by pipetting it into an empty sterile culture tube.
5. Another 1.0 mL of buffered detergent will be added for a second 30 second scrub.
6. The fluid from the second scrub will be removed and pooled with the fluid from the first scrub.

G. Disinfection of the Test Sites

After each test site is harvested, it will be disinfected with 70% isopropyl alcohol. When the harvesting of the last test site is completed, both forearms will be washed for approximately thirty (30) seconds with Hibiclens® (4% chlorhexidine gluconate). After the arms have been washed with Hibiclens®, a small amount of Polysporin® antibiotic ointment will be applied to each test site.

H. Plating and Incubation of the Organisms

Specimens from each of the four (4) sites will be plated within four (4) hours after harvesting. For plating, they will be serially diluted in half-strength (0.0375 M) buffer in ten-fold dilutions to 10^{-4} . 0.1 mL aliquots of each undiluted and diluted specimen will be pipetted onto the surface of duplicate plates, containing Trypticase Soy Agar with polysorbate (Tween) 80. The aliquots will be evenly spread on the surface of the plate with a sterilized bent glass rod. The media for these analyses are shown in Appendix F.

Plated samples will be incubated aerobically for 18 - 24 hours at $35 \pm 2^{\circ}\text{C}$. The CFU's of test bacteria will be counted at the end of the incubation period. In general, the number of CFU's per sample will be determined by taking the average of the counts from the plates which are in the range of ≥ 25 to ≤ 250 CFU's. If there are no plates with counts within this range, the following rules will be used to determine which counts will be used for the obtaining the number of CFU's for that specimen:

1. If all of the counts are below the prescribed range, the numbers below 25 from the undiluted plates will be used.
2. If the counts from the highest dilution are > 250 , the numbers, obtained from using the estimated counting procedure described in Appendix F, will be used.

The number of CFU's for each dilution counted will be recorded on Source Document 2.

V. Study Material and Instructions

A. Study Materials

All test products will be sent by the Sponsor to the clinical site prior to study initiation.

Each treatment product will be identified with the appropriate label (Appendix D) affixed to the outside of each container.

B. Shipping of Study Materials

The quantity of all materials, including test products and study supplies shipped to and returned from the clinical site, will be documented on the Shipping and Receiving Form (Source Document 1). The products will be packed into one or more cartons labeled with:

1. the study number;

2. distributor statement (i.e., "Distributed by Hill Top Research, Inc. with the test facility's full address and phone number); and
3. any applicable safety and handling procedures.

C. Return of Study Materials

Upon completion of the study, the Investigator will insure that all test products, whether completely used, partially used, or unused will be returned to the Sponsor at the following address:

The Procter & Gamble Company
Sharon Woods Technical Center
11511 Reed Hartman Highway
Cincinnati, Ohio 45241
Attn.: Jan Englehart

VI. Other Study Documentation and Requirements

A. Adverse Event and Intercurrent Event Reporting

Should any unexpected or serious adverse event occur during the clinical study or as a result of application of the test organism to the skin of the subjects, the subject will be requested to return to the site to be examined by the Investigator. The Investigator will determine whether: (a) the adverse event is likely to be associated with product treatment or the study procedures; (b) the event warrants termination of participation; and (c) to prescribe treatment, if necessary. The Investigator will notify the Sponsor representatives, Ward Billhimer, 513-626-1926 (work) or 513-831-8163 (home) or Jan Englehart, 513-626-1896 (work) or 513-385-9596 (home).

Each subject will need to be followed until the resolution of any adverse event. Information pertaining to the presenting signs, working diagnosis, assessment of the relationship of the adverse event to the product treatment, results of the follow-up visits and any prescribed treatment, will be documented in CRF 4. If treatment by a physician is necessary, this treatment will be documented on CRF 5.

B. Deviations from Protocol

Any deviations from the protocol that occur during execution and not previously agreed to by the Sponsor and Investigator will be documented. All changes in the protocol must be made in written amendments agreed upon by the Investigator and Sponsor. The amendments must be attached to the protocol on file.

C. Subject Termination and Completion

At the termination of the study, CRF 6 will be completed on all subjects. A concerted effort will be made to retain and follow all subjects in the study. Subjects, who terminate their own participation, prior to study closure, for any of the following reasons will also be documented in CRF 6.

- a) Intolerance of the study procedures.
- b) Intercurrent illness which interferes with the evaluation.
- c) Noncompliance with the protocol.
- d) Investigator decision to withdraw a subject from study.
- e) Subjects, who are prescribed medication for an illness arising during the study, may be terminated on the basis of an intercurrent event. This event will be

noted on the appropriate CRF's.

- f) Subjects who decide to withdraw from the study for personal reasons.

D. Investigator Review

The Investigator will review all case report forms and will sign the Investigator Review Form (CRF 6) at study termination attesting to the completeness and accuracy of case report forms that pertain to their responsibilities.

VII. Statistical Analyses

Data will be analyzed using analysis of variance. Data will be analyzed according to a 4 x 2 factorial design with factors of treatment, occlusion, and subject. Additional terms to be included in the model provided there are sufficient degrees of freedom are side, arm site, and the side by site interaction. P-values ≤ 0.10 will be considered significant.

VIII. Ethical and Regulatory Requirements

A. Institutional Review Board (IRB) Review and Approval

Review by an IRB is required to conduct this study. A copy of the approval letter along with a list of the IRB members who acted on this protocol and a statement that the IRB is in compliance with current Good Clinical Practices (GCP) regulations will be provided to the Sponsor.

B. Subject Informed Consent

Prior to study initiation, all subjects will be informed as to the type of study, the procedures to be followed, the general nature of the products being tested, and any known or anticipated adverse reactions which might result from participation. Each subject must sign the written informed consent (Appendix A) before participating in this study. The informed consent will contain all the basic elements outlined in 21 CFR 50.25.

C. Study Monitoring

The Investigator will permit a representative of the Sponsor to make regular visits during the course of the study. During these visits, the Investigator will permit the Sponsor's Monitor to inspect all forms and corresponding study subject's records to verify adherence to the protocol. The Sponsor's Monitor will also be permitted to review and verify laboratory reports, case report forms, drug/test article supply and inventory records. Any comments/instructions made by the Sponsor's Monitor will be recorded in the Investigator's study file.

D. Protocol Revisions and Amendments

With the exception of emergency situations, no changes or deviations from this protocol will be permitted without documented approval from the Investigator and the Sponsor's Monitor.

All amendments to the final protocol will be initiated by the Sponsor. They will be consecutively numbered, describe any changes being made, and the reasons for them. All amendments will be signed and dated by the Sponsor and the Investigator, and the impact on the study noted. If the Investigator deviates from the agreed final protocol, the Sponsor's Monitor will be informed of the change as soon as possible by telephone.

E. Final Report

The Sponsor will generate a final report of clinical results.

F. Study Safety Statement

The requested testing meets the ethical requirements stipulated in the Procter & Gamble Policy for Research Involving Human Subjects. Appropriate safety testing has been completed and risk assessments justify the placement of the test products in this study at these concentrations (levels of exposure).

G. Confidentiality

The obligations of the Investigator, Hill Top Research, Inc., regarding the confidential information on the antibacterial soap and all aspects of the study will be kept confidential according to the Laboratory Service agreement between Hill Top Research, Inc. and The Procter & Gamble Company.

IX. References

1. Williamson, P. and Kligman, A.M., A new method for the quantitative investigation of cutaneous bacteria. *J. Invest. Dermatol.*, 45:6 (1965) 498-503.

X. Sponsor and Investigator Concurrence

For The Procter and Gamble Company

PREPARED BY:

Jan S. Englehart
Jan S. Englehart, B.S., ASCP, Clinical Research Associate
Clinical Research and Biometrics Department

Date: 11/10/97

APPROVED BY:

Ward L. Billhimer
Ward L. Billhimer, M.S., Senior Scientist
Clinical Research and Biometrics Department

Date: 11/12/97

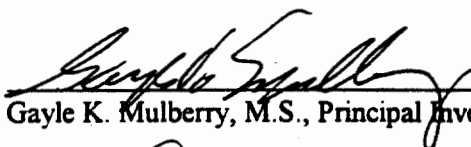
Paula B. Neumann
Paula B. Neumann, Ph.D., Senior Scientist Biostatistician
Clinical Research and Biometrics Department

Date: 11/18/97

Agreed and Accepted by Hill Top Research, Inc. and the Study Investigator for
CRB-97-12-262-CD:

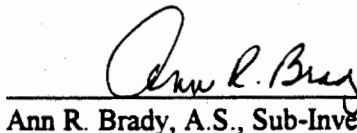
I certify that I have reviewed and approved the protocol, informed consent form, and other associated documents and agree to abide by their terms. In addition, I agree to conduct this clinical study in compliance with federal, state and local government regulations, guidelines and standards applicable to such studies including, but not limited to, those relating to Institutional Review Board (IRB), Informed Consent, and Good Clinical Practices.

I am aware that it is the responsibility of the Investigator to promptly report to the IRB all changes to the research activity and all unanticipated problems involving risk to human subjects. In addition, as Investigator, I am aware that a summary report must be submitted to the IRB when the study is completed. These guidelines are in accordance with CFR 312.66. The Sponsor will be copied on all correspondence to and from the IRB.



Gayle K. Mulberry, M.S., Principal Investigator

Date: 11-15-97



Ann R. Brady, A.S., Sub-Investigator

Date: 11.19.97

XI. Attachments

The following Appendices, Case Report Forms, and Source Documents are included as attachments to the Final Protocol:

Appendices

- A Written Informed Consent
- B Subject's Instructions Following Study Completion
- C Wash Procedure
- D Product Labels
- E List of Antibacterial/Antimicrobial Products
- F Microbiological Media

Case Report Forms

- 1 Demographics/Dermatological/Medical History Form
- 2 Inclusion/Exclusion Form
- 3 Treatment Record
- 4 Adverse Event
- 5 Physician's Action Report Form
- 6 Subject Termination and Investigator Review Form

Source Documents

- 1 Shipping and Receiving of Study Material
- 2 Microbiology Worksheet - Enumeration of Organisms

Appendix A

**HT# 97-5461-11
CRB-97-12-262-CD**

WRITTEN INFORMED CONSENT

To be provided by the clinical site.

Appendix B

**HT# 97-5461-11
CRB-97-12-262-CD**

SUBJECT'S INSTRUCTIONS FOLLOWING STUDY COMPLETION

You have just completed participation in a clinical study, "Residual Effectiveness Screening Test". During this study, two (2) test sites on each of your forearms were inoculated with *Escherichia coli* bacteria. Although we do not expect you have any adverse experience as a result of participation in this study, there is a remote possibility that an infection may develop on your forearms within the next 48 - 72 hours.

To determine whether you have developed an infection from the test bacteria, we would like you to examine your arms during the next 48 - 72 hour period. If you notice the appearance of any pimples, blisters, or raised red itching bumps surrounded by redness and/or swelling, please contact Gayle Mulberry or Ann Brady at (513) 831-3114 during normal business hours (8:15 am - 5 pm) or at (513) 831-3354 after hours.

Thank you for your cooperation.

Appendix C

**HT# 97-5461-11
CRB-97-12-262-CD**

WASH PROCEDURE

Water temperature should be maintained at 95 -100° F.

The temperature should be checked and recorded before each wash.

Water flow should be 4 L/minute.

Time of each wash should be recorded.

A technician will wash each subject's arm.

The technician will wear gloves for this procedure, changing after each treatment area wash.

Wipe the template with 70% isopropyl alcohol after use.

Begin with the subject's right arm:

- 1. Using the template, mark two (2) 10 x 5 cm treatment areas (an upper and lower) on the subject's forearm.**
- 2. The subject should wet the upper treatment site of their forearm under the running water.**
- 3. Dispense 0.5 mL of the appropriate test product, from a 1 cc disposable syringe, onto the upper treatment site area.**
- 4. The technician should wet their gloved hand under the running water.**
- 5. The technician should carefully lather the test product with two (2) fingers in an up-and-down motion within the upper treatment site for forty-five (45) seconds.**
- 6. The subject should rinse the upper treatment site avoiding crossover to the lower treatment site under the running water. Rinse for fifteen (15) seconds. Do not rub!**
- 7. Repeat steps 1 to 6 for the lower treatment site.**
- 8. Pat subjects' forearms dry using a paper towel. Do not rub!**
- 9. Repeat steps 1 to 8 on the left forearm.**

Appendix D
HT# 97-5461-11
CRB-97-12-262-CD

PRODUCT LABELS

**ANTIBACTERIAL
LIQUID SOAP PRODUCT
Product Code A**

Study# CRB-97-12-262-CD
HT#97-5461-11
Net Contents: 100 g
Distributed by:
Hill Top Research
Main and Mill Streets
Miamiville, Ohio 45147
(513) 831-3354
May Contain: Triclosan (TCS)
Exp. Date: 1/1/98
Use as directed for washing
arms only.

**ANTIBACTERIAL
LIQUID SOAP PRODUCT
Product Code G**

Study# CRB-97-12-262-CD
HT#97-5461-11
Net Contents: 100 g
Distributed by:
Hill Top Research
Main and Mill Streets
Miamiville, Ohio 45147
(513) 831-3354
May Contain: Triclosan (TCS)
Exp. Date: 1/1/98
Use as directed for washing
arms only.

**ANTIBACTERIAL
LIQUID SOAP PRODUCT
Product Code I**

Study# CRB-97-12-262-CD
HT#97-5461-11
Net Contents: 100 g
Distributed by:
Hill Top Research
Main and Mill Streets
Miamiville, Ohio 45147
(513) 831-3354
May Contain: Triclosan (TCS)
Exp. Date: 1/1/98
Use as directed for washing
and arms only.

**ANTIBACTERIAL
LIQUID SOAP PRODUCT
Product Code J**

Study# CRB-97-12-262-CD
HT#97-5461-11
Net Contents: 100 g
Distributed by:
Hill Top Research
Main and Mill Streets
Miamiville, Ohio 45147
(513) 831-3354
May Contain: Triclosan (TCS)
Exp. Date: 1/1/98
Use as directed for washing
and arms only.

Appendix E
HT# 97-5461-11
CRB-97-12-262-CD

LIST OF ANTIBACTERIAL / ANTIMICROBIAL PRODUCTS

Medicated Acne Cleansers

Benzac W Wash 5
Desuam-X 5 Wash
Benzac W Wash 10
Desquam-X 10m Wash
Fostex 10% BPO Wash
Oxy 10 Wash
Propa P.H. Liquid Acne Soap
PanOxyl 5
Fostex 10% BPO
PanOxyl 10
Clearasil Antibacterial Soap
Sastid Plain Therapeutic Shampoo and Acne Wash
Oxy Clean Soap
Fostex Medicated Cleansing Bar
Salicylic Acid and Sulfur Soap
Sulfur Soap

Antidandruff Shampoos

Head and Shoulders (all formulas)
Selsun Blue (all formulas)
Pert Plus for Dandruff
Suave for dandruff
Neutrogena T-gel
Neutrogena T-sal
Scalpacin
Tegrin
Any antidandruff shampoo

Anti-bacterial Soaps

Safeguard bar and liquid
Lever 2000 bar and liquid
Irish Spring bar
Dial bar and liquid
Softsoap Antibacterial Soap

Antibiotic Ointments and Creams

Bacitracin
Polysporin
J & J First Aid Cream
Neomycin

Anti-bacterial Dishwashing Liquids

Dawn
Joy
Palmolive
Dial

Appendix F
HT# 97-5461-11
CRB-97-12-262-CD

MICROBIOLOGICAL MEDIA

0.075M. Phosphate Buffer Solution

Weigh 0.4 grams of KH_2PO_4 , 10.1 grams of Na_2HPO_4 , 1.0 gram Triton X, 15.0 grams of polysorbate (Tween) 80, and 10.0 grams of Lecithin. Dissolve in 1 liter of distilled or deionized water. Adjust to pH 7.9 with 0.1N NaOH. Dispense buffer in 100 mL quantities in bottles. Loosely cap bottles and sterilize in the autoclave at 121°C. Prepared buffer is checked for sterility and stored at 15 - 30°C for upto 30 days.

Trypticase Soy Broth (TSB)

Dissolve 30 grams in 1 liter of distilled or deionized water. If necessary, warm slightly to dissolve completely. Dispense broth in 9 mL quantities in sterile tubes. Sterilize at 121°C. Check for sterility. Prepared tubes are stored at 15 - 30°C and used within 30 days.

Trypticase Soy Agar with Polysorbate (Tween) 80

Suspend 40 grams in 1 liter of distilled or deionized water in a heat resistant flask. Heat to boiling with gentle mixing to dissolve completely. Add 15 grams of polysorbate (Tween) 80 and gently mix to dissolve completely. Loosely cap flask and sterilize in the autoclave at 121°C. Cool to 45 - 50°C in a water bath. Pour in sterile 15 x 100 mm Petri dishes. Allow to cool and solidify on a level flat surface. Check for sterility. Prepared plates are stored at 2 - 8°C and used within 30 days.

Estimated Plate Count Procedure

Do not record counts on crowded plates from the highest dilution as too numerous to count (TNTC). If the number of colonies per plate exceeds 250, count colonies in those portions of the plate that are representative of colony distribution and calculate the Estimated Standard Plate Count (ESPC) from these counts. The ESPC will be determined utilizing the grid embossed area on the lighted surface of the colony counter. Each large square on the grid is 1 cm². If there are fewer than 10 colonies per square centimeter, count colonies in 12 squares, selecting, if representative, six consecutive squares horizontally across the plate and six consecutive squares at right angles, being careful not to count a square more than once. When there are more than 10 colonies per square centimeter, count colonies in four such representative portions. In both instances, multiply the average found per square centimeter by the area of the plate used to determine the estimated number of colonies per plate.

If the total number of CFU's have been estimated according to the procedure described above, ESPC (Estimated Standard Plate Count) should be recorded following the value.

Note: If the highest dilution plated contains >250 CFU's and a count ≤300 CFU's has been previously determined, that value may be reported. It will not be necessary to estimate the total CFU's on a plate containing >250 CFU's using the above procedure. Plates containing the highest dilution of test specimen plated and the CFU counts are greater than 300, then the above procedure should be used to determine the total CFU count.

Case Report Form 1
DEMOGRAPHICS/DERMATOLOGICAL/MEDICAL HISTORY FORM

Study #	Hill Top Research, Inc.	Visit Code	Date	Subject Initials	Subject Screen #
97-5461-11	Gayle K. Mulberry	Subject Qualification	____/____/____ mm dd yy	____/____/____ F M L	Permanent #:

Gender: <input type="checkbox"/> Male ⁽¹⁾ <input type="checkbox"/> Female ⁽²⁾	Age: _____ Years
---	------------------

Does the subject have any of the following at the treatment sites?

I. DERMATOLOGIC DISORDER	No	Yes	Don't Know
1. Psoriasis ?			
2. Eczema ?			
3. Skin Cancer ?			
4. Skin Allergies ? Please specify:			
5. Hives ?			

Does the Subject have any of the following (present and past)?

II. OTHER MEDICAL INFORMATION	No	Yes	Don't Know
1. Allergies ? Please specify.			
2. Hepatitis ?			
3. Heart and Vascular Disease?			
4. Liver Disease ?			
5. Kidney Disease ?			
6. Tuberculosis ?			
7. Diabetes ? Controlled? Diet [] Oral [] Insulin []			
8. Cancer ?			
9. Auto-immune disease (Lupus erythematosus, thyroiditis, AIDS, etc.) ?			
10. Organ transplant ?			
11. Any other condition not listed ? Please specify:			

Is the subject taking any medication? If yes, please specify below:

III. MEDICATION	No	Yes	Don't Know
1. Antibiotics, oral or systemic ?			
2. Cortisone, Steroids, ACTH, Anti-reaction Drugs ?			
3. Heart Medication ?			
4. Insulin ?			
5. Other ?			

Comments:

Based on the above medical history, the subject is: ☐ Qualified or ☐ Not qualified for the study.

Interviewer's Signature:	Date: ____/____/____ mm dd yy
--------------------------	----------------------------------

Case Report Form 2

INCLUSION / EXCLUSION FORM

Study #	Hill Top Research, Inc.	Visit Code	Date	Subject Initials	Subject Screen #
97-5461-11	Gayle K. Mulberry	Subject Qualification	____/____/____ mm dd yy	____/____/____ F M L	Permanent #:

INCLUSION CRITERIA

Check one

YES

NO

Subject:

		1. Is 18 to 65 years ?
		2. Has signed informed consent ?
		3. Is healthy as evidenced by responses on CRF 1 ?
		4. Has forearms that are free of dermatoses, cuts, lesions, and other skin disorders ?
		5. Is willing to comply with all study protocol requirements ?

EXCLUSION CRITERIA

Check one

YES

NO

N/A

Subject:

			1. Is currently participating in another clinical study at this or any other facility ?
			2. Has participated in any type of hand or arm wash study within the past 14 days ?
			3. Has cuts, lesions, or other skin disorders on the volar surface of either forearm ?
			4. Has soap, detergent, and/or perfume allergies ?
			5. Has eczema or psoriasis on their arm(s) ?
			6. Has taken systemic antibiotics or used topical antibiotics for any reason in the 3 weeks prior to the start of the test period ?
			7. Are currently using antibacterial/antimicrobial soaps (liquids and/or bars), medicated lotions and creams, and/or anti-dandruff shampoos ?
Female	Female	Male	8. Is currently pregnant ? <input type="checkbox"/> Yes <input type="checkbox"/> No Of child-bearing potential: <input type="checkbox"/> Yes <input type="checkbox"/> No <input type="checkbox"/> Surgically Sterile <input type="checkbox"/> Post-menopausal If of child bearing potential - β -HCG Test Results: <input type="checkbox"/> negative <input type="checkbox"/> positive
Female	Female	Male	9. Is currently lactating?
			10. Has been medically diagnosed as having a medical condition such as: diabetes, hepatitis, an organ transplant, or AIDS (or HIV positive) ?
			11. Has another medical condition which in the opinion of the Investigator would preclude participation ?

Based upon dermatologic evaluation and the information contained in Case Report Forms 1 and 2, the subject is:

☐ **Qualified**☐ **Not Qualified**

for participation in this study.

Reasons for disqualification: _____

Investigator's Signature: _____

Date: / /
mm dd yy

**Case Report Form 3
TREATMENT RECORD**

Study #	Hill Top Research, Inc.	Visit Code	Subject Initials	Permanent #:
97-5461-11	Gayle K. Mulberry	Test Period	____/____/____ F M L	

WASH RECORD						
Wash	Right Arm Test Sites		Left Arm Test Sites		Water Temp.	Initials / Date
	Upper (1)	Lower (2)	Upper (3)	Lower (4)		
Product Code						
1	am / pm	am / pm	am / pm	am / pm		/

INOCULATION, OCCLUSION, AND HARVESTING						
Right Arm	Site	Time Period	Inoculation Time	Occluded	Harvesting Time	Initials / Date
	<i>upper (1)</i>		am / pm	yes / no	am / pm	/
	<i>lower (2)</i>		am / pm	yes / no	am / pm	/
Left Arm	Site	Time Period	Inoculation Time	Occluded	Harvesting Time	Initials / Date
	<i>upper (3)</i>		am / pm	yes / no	am / pm	/
	<i>lower (4)</i>		am / pm	yes / no	am / pm	/

Microbiologist's Initials:	Investigator's Signature:	Date: ____/____/____ mm dd yy
----------------------------	---------------------------	----------------------------------

Case Report Form 4

ADVERSE EVENT

Study #	Hill Top Research, Inc.	Date	Subject Initials	Permanent #:
97-5461-11	Gayle K. Mulberry	____/____/____ mm dd yy	____/____/____ F M L	

Was reaction related to treatment? ☐ Not related ☐ Possibly related ☐ Definitely related ☐ Other (explain)

Did subject take any medication during the study period? ☐ YES ☐ NO If yes, complete section below.

Date of Onset: _____ Date Reported: _____ Date Resolved: _____

Describe event: _____

Action Taken: ☐ None ☐ Continued on study ☐ Withdrawn from the study ☐ Consulted physician

☐ Medication taken (Complete below) ☐ Hospitalized ☐ Other (explain)

Additional Comments:

FOLLOW - UP ACTION TAKEN

Date	Action Taken	Comments	Initials

CONCOMITANT MEDICATION TAKEN

Medication (Oral or Systemic)	Total Daily Dose	Start Date mm / dd / yy	Stop Date mm / dd / yy	Indication (Reason for Taking)
		/ /	/ /	
		/ /	/ /	
		/ /	/ /	

Investigator's Signature:

Recorded by:

Date

____/____/____
mm dd yy

Case Report Form 5

PHYSICIAN'S ACTION REPORTING FORM

Study #	Hill Top Research, Inc.	Date	Subject Initials	Permanent #:
97-5461-11	Gayle K. Mulberry	____/____/____ mm dd yy	____/____/____ F M L	

Date(s) of office visit(s): _____

Pertinent Medical History: (e.g., causes of similar reactions, known allergies,
potential involvement of current medications or medical conditions)

Test Product Exposure:

Use Began On: _____ Date Used Ended on: _____ Date Number of Uses: _____

Clinical Observations: (Include date of onset and descriptions/severity/locations, etc.)

Impression: _____

Treatment: _____

Follow Up: _____

Date Resolved: _____

Is condition related to use of the test products?

☐ Probably related* ☐ Not Related* ☐ Unknown

Reasons: _____

Physician's Signature_____
Date

Case Report Form 6

SUBJECT TERMINATION AND INVESTIGATOR REVIEW FORM

Study #	Hill Top Research, Inc.	Visit Code	Subject Initials	Permanent #:
97-5461-11	Gayle K. Mulberry	End of Study	____/____/____ F M L	

Date Subject Entered the Study: ____/____/____ mm dd yy	Date Subject Withdrew / Completed the Study: ____/____/____ mm dd yy
---	--

Did subject drop out of study prior to completion? ☐ YES ☐ NO If yes, complete below:

Check reason for subject premature termination:

- ☐ 1 = Adverse Event (Documented on CRF 4)
- ☐ 2 = Intercurrent Event
- ☐ 3 = Lack of Compliance with Protocol Specify: _____
- ☐ 4 = Personal reasons (family problems, lack of transportation, etc.)
- ☐ 5 = No show, Lost to Follow-up
- ☐ 6 = Other Specify: _____

Comments: _____

**MY SIGNATURE ON THIS PAGE CERTIFIES
THAT I HAVE REVIEWED ALL OF THE DATA
AND STATEMENTS SUBMITTED FOR THIS
SUBJECT AND FIND THEM ACCURATE AND
COMPLETE TO THE BEST OF MY KNOWLEDGE.**

Investigator's Signature	Date ____/____/____ mm dd yy
--------------------------	------------------------------------

SOURCE DOCUMENT 1
CRB-97-12-262-CD
(HT# 97-5461-11)
SHIPPING & RECEIVING OF STUDY MATERIAL

MATERIALS SHIPPED		MATERIALS RECEIVED	MATERIALS RETURNED	MATERIALS RECEIVED
Date Shipped: _____ Time: _____		Date Received: _____	Date: _____ Time: _____	Date Received: _____
Test Site: _____		Time: _____	Test Site: _____	Time: _____
Method of Transport: _____		Test Site: _____	Method of Transport: _____	Test Site: _____
LISTING OF MATERIALS SHIPPED		MATERIALS RECEIVED	MATERIALS RETURNED	MATERIALS RETURNED
# of ITEMS	ITEM DESCRIPTION * * Include Batch no. in item description	4= OK D = LOST/DAMAGED NR = NOT RECEIVED	Indicate if items are returned or not returned	4= OK D = LOST/DAMAGED NR = NOT RECEIVED

Shipping Technician: _____ Date: _____	Receiving Technician: _____ Date: _____	Returning Technician: _____ Date: _____	Receiving Technician: _____ Date: _____
---	--	--	--

SOURCE DOCUMENT 2

HT# 97-5461-11

CRB-97-12-262-CD

MICROBIOLOGY WORKSHEET - ENUMERATION OF ORGANISMS

CFU COUNTS (TOTAL # ORGANISMS / mL)									
Date Plated:			Date Counted:				Inoculum Count:		
Subject No.	Test Site	Right Arm				Left Arm			
		1 (upper)		2 (lower)		3 (upper)		4 (lower)	
		Plate 1	Plate 2	Plate 1	Plate 2	Plate 1	Plate 2	Plate 1	Plate 2
	10 ⁻¹								
	10 ⁻²								
	10 ⁻³								
	10 ⁻⁴								
	10 ⁻⁵								
Subject No.	Test Site	Right Arm				Left Arm			
		1 (upper)		2 (lower)		3 (upper)		4 (lower)	
		Plate 1	Plate 2	Plate 1	Plate 2	Plate 1	Plate 2	Plate 1	Plate 2
	10 ⁻¹								
	10 ⁻²								
	10 ⁻³								
	10 ⁻⁴								
	10 ⁻⁵								
Subject No.	Test Site	Right Arm				Left Arm			
		1 (upper)		2 (lower)		3 (upper)		4 (lower)	
		Plate 1	Plate 2	Plate 1	Plate 2	Plate 1	Plate 2	Plate 1	Plate 2
	10 ⁻¹								
	10 ⁻²								
	10 ⁻³								
	10 ⁻⁴								
	10 ⁻⁵								
Subject No.	Test Site	Right Arm				Left Arm			
		1 (upper)		2 (lower)		3 (upper)		4 (lower)	
		Plate 1	Plate 2	Plate 1	Plate 2	Plate 1	Plate 2	Plate 1	Plate 2
	10 ⁻¹								
	10 ⁻²								
	10 ⁻³								
	10 ⁻⁴								
	10 ⁻⁵								
Laboratory Supervisor			Microbiologist				Date		

PROTOCOL AMENDMENT Issue I 11/24/97

Residual Effectiveness Screening Test of Antibacterial Liquid Products against *E. coli*
under Occluded and Unoccluded Conditions

Study Number: CRB-97-11-262-CD (HT 97-5461-11)

Purpose of the
Amendment: Changes to the Study Protocol

CHANGE #1 Clarification of the Title of the Protocol

The title of the protocol will be modified to read as follows:

**"Residual Effectiveness Screening Test of Liquid Products against *E. coli* under
Occluded and Unoccluded Conditions**

The word "antibacterial" has been removed since all of the test products do not contain the antibacterial active ingredient.

CHANGE #2 Clarification of the Objective of the Protocol

The objective of the protocol will be modified to read as follows:

"The objective of this study is to evaluate the residual effectiveness of four (4) liquid soap products against potentially pathogenic bacteria (*Escherichia coli*, ATCC 11229) under simulated skin conditions which are considered optimal for bacterial growth, proliferation, and possible infection."

The words "containing an antibacterial active" have removed since all of the test products do not contain the antibacterial active ingredient.

For The Procter and Gamble Company:

J. S. Englehart
J. S. Englehart, B.S., Clinical Research Associate

Date: 11/24/97

For Hill Top Research, Inc.:

Gayle K. Mulberry
Gayle K. Mulberry, M.S., Principal Investigator

Date: 11/24/97

Ann R. Brady
Ann R. Brady, A.S., Sub-Investigator

Date: 11.24.97

G

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Clinical Pharmacokinetics of the Salicylates

Christopher J. Needs and Peter M. Brooks

Department of Rheumatology, Royal North Shore Hospital, St Leonards, N.S.W.

Summary

The use of salicylates in rheumatic diseases has been established for over 100 years. The more recent recognition of their modification of platelet and endothelial function has led to their use in other areas of medicine.

Aspirin (acetylsalicylic acid) is still the most commonly used salicylate. Administration as an aqueous solution aspirin is rapidly absorbed at the gastric mucosa. Less rapid absorption is observed with other formulations due to the limiting step of tablet disintegration - this latter factor being maximal in the rate of gastric emptying. The rate of aspirin absorption is dependent not only on the formulation but also on the rate of gastric emptying.

Aspirin absorption follows first-order kinetics with an absorption half-life of 5 to 16 minutes. Hydrolysis of aspirin to salicylic acid by nonspecific esterases in the liver and, to a lesser extent, the stomach so that only 68% of the dose enters systemic circulation as aspirin. Both aspirin and salicylic acid are bound to serum proteins (aspirin being capable of irreversibly acetylating many proteins), and both are found in the synovial cavity, central nervous system, and saliva.

The serum half-life of aspirin is approximately 20 minutes. The fall in plasma concentration is associated with a rapid rise in salicylic acid concentration. Salicylic acid is renally excreted in part unchanged and the rate of elimination is influenced by pH, the presence of organic acids, and the urinary flow rate. Metabolism of salicylic acid occurs through glucuronide formation (to produce salicyl acyl glucuronide and salicyl phenolic glucuronide), conjugation with glycine (to produce salicyluric acid), and to gentisic acid. The rate of formation of salicyl phenolic glucuronide and salicyluric acid are easily saturated at low salicylic acid concentrations and their formation follows Michaelis-Menten kinetics. The other metabolic products follow first-order kinetics. The serum half-life of salicylic acid is dose-dependent; thus, the larger the dose the longer it will take to reach steady-state. There is also evidence that enzymatic salicyluric acid formation occurs.

No significant differences exist between the pharmacokinetics of the salicylates in the elderly or in children when compared with young adults. Apart from differences in the distribution of albumin-bound salicylate in various disease states and physiological states associated with low serum albumin, pharmacokinetic parameters in patients with rheumatoid arthritis, osteoarthritis, chronic renal failure or liver disease are the same. Pharmacokinetic interactions with various non-steroidal anti-inflammatory drugs do occur, but the clinical relevance of these is uncertain. Clinically important interactions may occur with heparin or oral anticoagulants, but these are due mainly to effects on platelet function rather than on pharmacokinetic parameters.

Pharmacokinetics of Sa

The salicylates have been used for a variety of conditions. Many medieval physicians and Greenberg and Greenberg and Greenberg on the use of a bark as an antipyretic isolated from the willow. The first synthesis of salicylic acid (ASA) was not until 1893 that the Bayer Pharmaceutical Company commercialized it into wide use.

Today the salicylates are available in many different forms (Table 1). Annual consumption is in the billions. Although one of the first drugs of first choice for rheumatic diseases has been replaced by non-steroidal anti-inflammatory drugs, it still plays a major role in the treatment of these diseases. Since the work of Willis (1971) and Vane (1971) on the role of prostaglandin synthetase (cyclo-oxygenase), its pharmacological role in inflammation (Mancini et al., 1979) but also in vascular disease (Ginsburg et al., 1978), coronary artery disease (Ginsburg et al., 1978), and arterial hypertension (Ginsburg et al., 1978) many of these conditions of aspirin has not been fully appreciated. It is amazing that only a few pharmacokinetic studies with some aspects in this article will be reviewed in the knowledge of the salicylates.

1. Physicochemical

Salicylic acid is a weak acid (pKa 3.5) and the salicylate ion is the predominant form at physiological pH.

Brooks

pharmacokinetics of the salicylates in healthy adults. Apart from differences in disease states and physiological conditions, kinetic parameters in patients with renal or liver disease are essentially the same as in healthy subjects. The use of non-steroidal anti-inflammatory drugs is certain. Clinically important interactions are few, but these are due mainly to altered pharmacokinetics.

Today the salicylates are available in hundreds of different forms (Buchanan et al., 1979) and the annual consumption of tablets can be measured in billions. Although over the last 25 years their place as drugs of first choice for the treatment of rheumatic diseases has been challenged by a variety of new non-steroidal anti-inflammatory agents, they still play a major role in the treatment of rheumatic diseases. Since the demonstration by Smith and Willis (1971) and Vane (1971) that aspirin prevented prostaglandin formation by inhibition of cyclo-oxygenase, it has been widely used as a pharmacological tool, not only in modifying inflammation (Moncada and Vane, 1979; Patrono et al., 1979) but also in the prophylaxis of cerebrovascular disease (Canadian Co-operative Study Group, 1978), coronary artery disease (Mustard, 1982), and arterial thrombosis (Genton, 1982). In many of these conditions the most appropriate dose of aspirin has not yet been determined and it is amazing that only in the last decade have the complex pharmacokinetics of aspirin been appreciated, with some aspects still unresolved (Levy, 1981). In this article we review the current state of knowledge of the clinical pharmacokinetics of the salicylates.

Salicylic acid (2-hydroxybenzoic acid) and aspirin (the salicylate ester of acetic acid) are both

In view of the labile nature of aspirin, great care must also be taken in collecting and processing biological fluids. Blood samples should be collected with fluoride and the plasma taken off immediately and frozen (Rowland and Riegelman, 1967). Even in dry ice, aspirin has a hydrolysis half-life of 24 days (Walter et al., 1974) and therefore analysis should be completed as rapidly as possible.

As can be seen from figure 1, the total plasma salicylate includes the sum of all the metabolites plus the parent compound. (These compounds will also be seen in the urine.) A variety of methods for measuring plasma, serum or urinary salicylate have been developed. The early methods were spectrophotometric using either Trinder's (1954) original method or a modification of this (Schachter and Manis, 1958). This employs the chelation of iron by salicylic acid or its analogues to yield a coloured complex whose absorbence is then measured photometrically. However, there are a number of errors in this method as the reagent shows poor specificity for salicylate and depends on the differential hydrolysis of aspirin to salicylic acid. To measure total salicylates in biological fluids they must be hydrolysed to convert all the salicylate to salicylic acid prior to the reaction with Trinder's reagent.

Chromatographic methods using gas-liquid chromatography have also been developed (Rowland and Riegelman, 1967; Walter et al., 1974), and a number of high-performance chromatographic (HPLC) methods have recently been described (Cham et al., 1980, 1982; Day et al., 1981; Peng et al., 1978; Rumble et al., 1981). These methods allow for rapid quantification of aspirin as well as

salicylic acid and the major metabolites (without the use of hydrolysis or derivitisation procedures) and have greatly enhanced our understanding of aspirin pharmacokinetics in body tissues.

3. Fundamental Pharmacokinetic Properties

3.1 Absorption

After oral administration, absorption of salicylate occurs rapidly by passive diffusion of unionised lipophilic molecules from the stomach (Hogden et al., 1957; Rowland et al., 1972). Extensive salicylate absorption also occurs in the jejunum and small bowel by virtue of its surface area. A passive process again appears responsible for small bowel salicylate absorption (Hogden et al., 1959; Schanker et al., 1958). Although aspirin can spontaneously hydrolyse, this is slow so that there is little or no free salicylate in the intestine and it is absorbed as aspirin rather than salicylic acid (Leonards, 1962).

Rectal absorption of salicylate is also possible. Cutaneous absorption may occur from salicylate containing rubefacients (Davison, 1971).

Following oral administration of an aqueous solution, the absorption kinetics of aspirin were found to follow a first-order process (Rowland et al., 1972). In this study, 68% of the dose reached the systemic circulation unmetabolised, although there was wide variation in absorption half-life (from 4.5 to 16 minutes). The remainder of the dose was considered to have been metabolised during passage from gastrointestinal fluids to the systemic circulation by esterases in the gut wall, plasma or liver.

Plasma concentrations of aspirin rise rapidly following absorption, with peak concentrations occurring approximately 25 minutes after ingestion of soluble aspirin preparations (Morgan and Rowland, 1965; Rowland et al., 1972) or 4 to 6 hours after ingestion of enteric-coated aspirin (Rowland et al., 1982). Plasma aspirin concentrations decrease

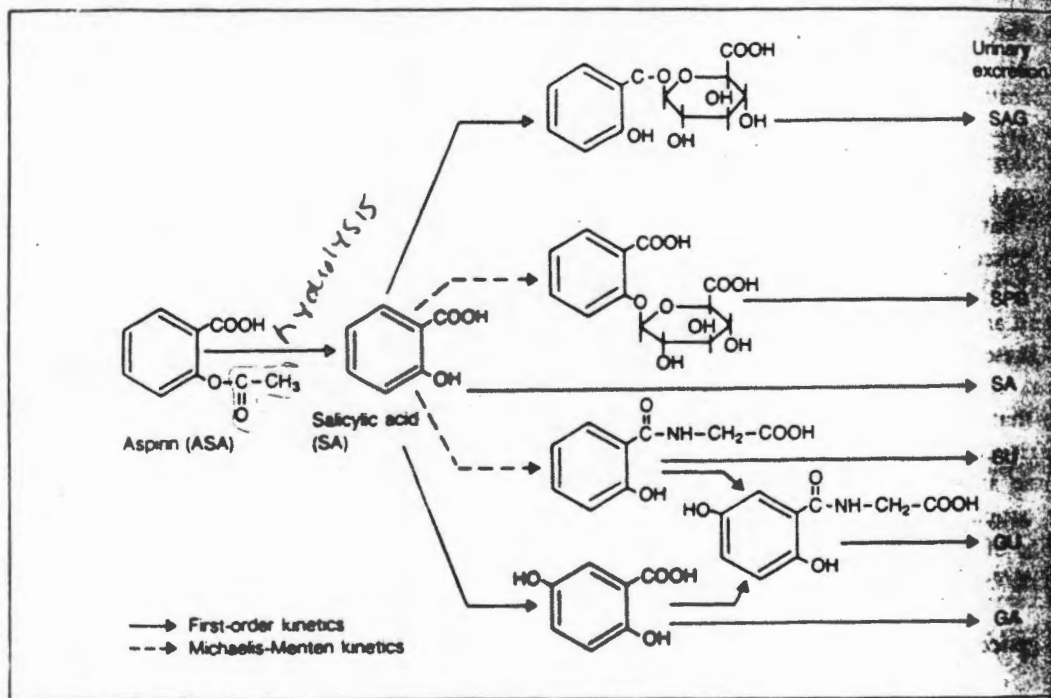


Fig. 1. Salicylate metabolism. (SAG = salicyl acyl glucuronide; SPG = salicyl phenolic glucuronide; SU = salicyl succinate; GA = salicyl gentisate; GU = gentisic acid)

after achieving peak concentrations in the blood.

The factors affecting the absorption of aspirin are shown in table I. Absorption of aspirin is increased at higher pH. The peak salicylate concentration is different between pH 1 and pH 8 (Leonards, 1976) due to the higher pH. Food can also reduce the rate of absorption of aspirin (Volans, 1974). Aspirin is metabolised during passage from gastrointestinal fluids to the systemic circulation by esterases in the gut wall, plasma or liver.

Plasma concentrations of aspirin rise rapidly following absorption, with peak concentrations occurring approximately 25 minutes after ingestion of soluble aspirin preparations (Morgan and Rowland, 1965; Rowland et al., 1972) or 4 to 6 hours after ingestion of enteric-coated aspirin (Rowland et al., 1982). Plasma aspirin concentrations decrease rapidly after achieving peak concentrations in the blood. The factors affecting the absorption of aspirin are shown in table I. Absorption of aspirin is increased at higher pH. The peak salicylate concentration is different between pH 1 and pH 8 (Leonards, 1976) due to the higher pH. Food can also reduce the rate of absorption of aspirin (Volans, 1974). Aspirin is metabolised during passage from gastrointestinal fluids to the systemic circulation by esterases in the gut wall, plasma or liver.

Plasma concentrations of aspirin rise rapidly following absorption, with peak concentrations occurring approximately 25 minutes after ingestion of soluble aspirin preparations (Morgan and Rowland, 1965; Rowland et al., 1972) or 4 to 6 hours after ingestion of enteric-coated aspirin (Rowland et al., 1982). Plasma aspirin concentrations decrease rapidly after achieving peak concentrations in the blood.

Salicylate is also possible. Absorption may occur from the gastrointestinal tract (Davison, 1971). Administration of an aspirin tablet follows a first-order process. In this study, 68% of the aspirin was in the systemic circulation. There was wide variation in the time to peak (from 4.5 to 16 minutes). The time to peak was considered to be the time of passage from gastrointestinal circulation by esterases in the stomach or liver. The concentrations of aspirin rise rapidly, with peak concentrations usually 25 minutes after administration of preparations (Morgan and Morgan, 1972) or 4 to 6 hours for enteric-coated aspirin (Ross and Ross, 1972). Aspirin concentrations decrease rapidly after achieving peak values as plasma salicylic acid concentrations increase.

The factors affecting absorption of salicylate are shown in table I. Although gastric absorption of aspirin is increased at low pH, the time to reach peak salicylate concentrations is not significantly different between pH 5 and pH 3 (Dotevall and Elvened, 1976) due to increased gastric emptying at the higher pH. Food has been shown to significantly reduce the rate of absorption of effervescent aspirin (Volans, 1974), enteric-coated aspirin (Paull et al., 1976), and sustained-release forms (Brooks et al., 1978), while posture and activity may also affect gastric emptying at the time of drug ingestion. A number of studies (Castleden et al., 1977; Cuny et al., 1979; Salem and Stevenson, 1977) have failed to demonstrate significant differences in the absorption of salicylate between young and elderly subjects.

Aspirin (pK_a 3.5) and salicylic acid (pK_a 3.0) are weak acids, being 99% unionised at pH 1 and able to diffuse through lipid membranes. Schachter and Manis (1958) have shown that as the pH rises, the amount of salicylate absorbed from the stomach decreases. When salicylate is ingested in tablet form, it is the dissolution rate of the tablet that influences the rate of absorption (Levy and Hollister, 1965). The dissolution rate increases as the pH rises, and is maximal at pH 8 (Gibaldi, 1977). Salicylate salts are generally more soluble than the parent compounds (Leonards, 1963), with the exception of aluminium salts which are poorly absorbed (Levy and Sahli, 1962). Buffered aspirin preparations form salts as the tablets disintegrate giving enhanced dissolution and absorption (Levy and Hayes, 1960). Thus, the major factor affecting the absorption rate of aspirin or salicylic acid is the drug formulation itself (Martin, 1971).

The formulation of salicylate should, however, only influence the rate of absorption, such that the areas under the plasma concentration-time curves (AUCs) with each formulation will be similar. The most rapid absorption is obtained with effervescent tablets (Volans, 1974); buffered preparations containing 16 mmol of buffer are absorbed more rapidly than those containing 32 mmol (Mason and

Table I. Factors affecting salicylate absorption

Rate of gastric emptying
volume of food
pH of stomach contents
nervous state
concurrent drugs
exercise
posture
Formulation
Disease states associated with altered gastrointestinal transit time

Winer, 1981). Aspirin from enteric-coated tablets is completely absorbed but absorption is often delayed due to prolonged gastric emptying (Leonards and Levy, 1965; Siebert et al., 1983); concurrent use of metoclopramide will enhance the rate of absorption from enteric-coated tablets (Paull et al., 1976).

Although early enteric-coated preparations were associated with poor bioavailability, recent enteric-coated and sustained-release preparations demonstrate salicylate bioavailability in excess of 90% (Brooks et al., 1978; Day et al., 1976).

When given in suppository form rectal absorption kinetics are similar to those of oral gelatin capsules containing sodium salicylate, providing the pharmaceutical formulation of the suppository base allows complete aspirin release (Parrott, 1971).

3.2 Distribution

Once absorbed, salicylates are distributed extensively through body fluids. Reported values for the apparent volume of distribution (V_d) of salicylate range from 9.6 to 12.7L in adults (Graham et al., 1977), with similar values (0.12 to 0.14 L/kg) in children (Wilson et al., 1982).

3.2.1 Plasma Protein Binding

Both aspirin and salicylic acid are partially bound to serum proteins, primarily albumin (Reynolds and Cluff, 1960). It has been suggested that the binding of salicylic acid to albumin occurs mainly at 2 primary, and a number of secondary

Urinary excretion

SAG

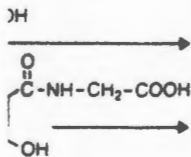
SPG

SA

SU

GU

GA



acurionide. SU = salicyluric

binding sites (Borgå et al., 1976). At therapeutic concentrations (1.1-2.2 mmol/L), salicylic acid is in molar excess compared with albumin such that binding is strongly dependent on both the salicylic acid and the albumin concentrations. The normal protein binding value of salicylic acid at therapeutic concentrations is 80 to 90% (Wanwimolruk et al., 1982). As the plasma concentration increases, the non-protein bound (free) fraction increases. Aspirin has been shown to acetylate the minor group of the 199 lysine residue in the human serum albumin primary sequence (Pinckard et al., 1968) and there is some evidence that this site is also shared by salicylic acid leading to a binding interaction between the two compounds (Ali and Routh, 1969; Pinckard et al., 1968). There is some evidence that salicylic acid binds to erythrocytes as well as to serum proteins (McArthur et al., 1971), but aspirin does not bind to proteins in the same reversible manner as it permanently acetylates the protein molecule (Hawkins et al., 1968). Protein acetylation is considered to be a major mechanism of action of aspirin leading to inactivation of enzymes such as prostaglandin synthetase (Hawkins et al., 1968; Pinckard et al., 1968; Roth and Chester, 1978).

Using a fluorescent probe analysis, Wanwimolruk et al. (1982) have recently failed to demonstrate any differences in the binding of salicylic acid to serum albumin in patients with rheumatoid arthritis or osteoarthritis.

3.2.2 Synovial Fluid Distribution

It has been shown that the protein binding of salicylic acid in synovial fluid is considerably lower than in plasma (Rosenthal et al., 1964; Soren, 1979; Trnavska and Trnavska, 1980; Wanwimolruk et al., 1983). Although the total salicylic acid concentration in synovial fluid is lower than that in plasma, this is likely to be explained by the lower albumin concentrations found in synovial fluid. Aspirin concentrations in synovial fluid are also significantly lower, but peak much later, than those concentrations seen in plasma, and aspirin remains in the synovial fluid long after it has disappeared from the plasma (Soren, 1979).

3.2.3 Cerebrospinal Fluid Penetration

Both salicylic acid and aspirin have been found to diffuse slowly into the cerebrospinal fluid due to the high degree of ionisation of salicylic acid at the pH (7.4) of plasma (Brodie et al., 1961). The ratio of CSF to plasma salicylic acid is less than would be predicted on the basis of pH alone, and the possibility of an active transport mechanism into the CSF of cats was suggested by Lorenzo and Spector (1973). These data have been confirmed in other *in vivo* studies (Lorenzo and Lorenzo, 1973), but only low salicylic acid concentrations were studied. A recently developed pharmacokinetic model has been used successfully to study salicylate kinetics in the CSF (Chen, 1978). Plasma pH is the major factor determining salicylic acid concentrations in the CSF. At the plasma pH, the more salicylic acid is present in the plasma, the more salicylic acid is found in the CSF (Goldberg et al., 1961).

3.2.4 Distribution in Saliva

In saliva, the concentration of salicylic acid has been found to be proportional to the plasma concentration (Brooks et al., 1978; Graham and Land, 1972; Roberts et al., 1978). However, concentration may vary with salivary site of production and it has been suggested that this method is unsuitable for routine concentration monitoring (Levy et al., 1978).

3.2.5 Placental Transfer and Secretion into Breast Milk

Salicylic acid readily crosses the placenta, plasma concentrations being higher at birth than concurrent maternal concentrations (Garnett *et al.*, 1975; Levy and Garrettson, 1973; Levy *et al.*, 1975). Salicylate distributes readily into breast milk and although after a single dose the amount ingested by a nursing infant is small, considerable exposure to salicylate is possible if the infant regularly ingests large doses (Findlay *et al.*, 1975).

3.3 Metabolism and Excretion

Aspirin is rapidly converted to salicylic acid with a half-life of only 15 to 20 minutes (Rowland and Tozer, 1995).

Pharmacokinetics of Sal

Riegelman, 1968). The specific esterases found in aspirin after oral and gastric mucosal administration after passing (Rainsford et al., 198

During absorption the gastrointestinal rates 28 to 35% of the (Rainsford et al., 1972). The mucosal cells of the stomach and duodenum and disease differ in enzyme activities (Gupta et al., 1972; Rainsford et al., 1974); for example, reduced in patients with ulcer (Rainsford et al., 1974). The form of the drug in the blood 15 minutes after ingestion is 10 minutes before the peak of gastric acid. However, the blood rapidly and the half-life is remarkably dependent on the dose. Diurnal variations have also been described (Semenowicz, 1979).

Salicylic acid is partly metabolised (see below). Free salicylic acid di-

Table II. First-order rate

Reference	No. of patients
Levy and Tsuchiya (1972)	4
Bochner et al. (1981)	5
Boreham and Mann (1969)	6

Abbreviations: k = first-
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Riegelman, 1968). This hydrolysis is due to non-specific esterases found in many body tissues (Harris and Riegelman, 1967). The acetyl component of aspirin after oral and intravenous dosing is found in gastric mucosal cells or is excreted as carbon dioxide after passing through the Krebs cycle (Rainsford et al., 1983).

During absorption, aspirin esterase activity in the gastrointestinal mucosal membranes contributes 28 to 35% of the hydrolysis of aspirin (Rowland et al., 1972). This esterase activity is highest in the mucosal cells of the gastric fundus (Dawson and Pryse-Davies, 1963), though considerable age, sex and disease differences may exist in tissue esterase activities (Gupta and Gupta, 1977; Menguy et al., 1972; Rainsford et al., 1980; Windorfer et al., 1974); for example, aspirin esterase activity is reduced in patients with alcoholic liver disease (Rainsford et al., 1980). Aspirin is the dominant form of the drug in the plasma during the first 20 minutes after ingestion and can be detected for several minutes before there is any measurable salicylic acid. However, it then disappears from the blood rapidly and hence the aspirin concentration is remarkably dependent on the rate of absorption. Diurnal variations in aspirin pharmacokinetics have also been demonstrated (Markiewicz and Semenowicz, 1979).

Salicylic acid is partly excreted unchanged and partly metabolised (see fig. 1 and section 3.4 below). Free salicylic acid diffuses readily across the glom-

erulus and is also actively secreted by the proximal tubule. The conjugates of salicylic acid are also renally excreted, being dependent on glomerular filtration and tubular secretion. The hydroxylated metabolite gentisic acid is excreted in the same way as free salicylic acid.

3.4 Elimination Kinetics

After intravenous administration of aspirin, its elimination kinetics are described by a biexponential equation, the half-life of the second component being 13 to 19 minutes (Rowland and Riegelman, 1968). *In vitro* hydrolysis of aspirin to salicylic acid by esterase in the plasma is rapid, with a half-life of 15 to 20 minutes (Rowland et al., 1972).

Salicylic acid is removed from the body by 5 parallel and competing pathways, namely, renal elimination and formation of 4 metabolites (fig. 1). Salicylic acid is conjugated with glycine to form salicyluric acid (SU), conjugated with glucuronic acid to form salicyl phenolic glucuronide (SPG) and salicyl acyl glucuronide (SAG), and oxidised to gentisic acid (GA) [Levy and Tsuchiya, 1972]. Gentisuric acid (GU) may be formed from SU via microsomal oxidation or from GA via glycine conjugation (Wilson et al., 1978). The elimination pathways of salicylic acid to SU and SPG are saturable and follow Michaelis-Menten kinetics, whereas the other pathways exhibit linear (first-order) kinetics (Levy, 1965, 1971, 1979; Levy and

Table II. First-order rate constants and Michaelis constants reported in various studies of salicylate elimination kinetics

Reference	No. of patients	SA k_e (h ⁻¹)	SA→SAG k (h ⁻¹)	SA→GA k (h ⁻¹)	SA→SU		SA→SPG	
					V_{max} (mg/h)	K_m	V_{max} (mg/h)	K_m
Levy and Tsuchiya (1972)	4	0.0075	0.0071	0.0023	60.3	338 (mg)	32.3	629 (mg)
Bochner et al. (1981)	5				43.4 ± 10		14.3 ± 3.4	
							(mg/L)	
Boreham and Martin (1969)	6			0.006				

Abbreviations: k = first-order rate constant; k_e = elimination rate constant; K_m = Michaelis-Menten constant; V_{max} = theoretical maximum velocity of metabolism.

Leonards, 1966; Levy et al., 1972; Tsuchiya and Levy, 1972b). The rate constants applying to these processes are shown in table II.

After a small dose of aspirin (300mg or less), about 90% is excreted as SU or SPG (Levy and Leonards, 1966). Levy and co-workers have demonstrated that while steady-state plasma salicylic acid concentrations increase with increasing doses, steady-state concentrations of SU do not. After a 3g dose of aspirin, 50% of the dose was excreted as SU, 20% as SPG, 14% as salicylic acid, 11.0% as SAG, and 3.1% as GU (Levy et al., 1972; Tsuchiya and Levy, 1972a). The saturable nature of the SU pathway, and the V_{max} and K_m values have been elegantly defined by utilising the inhibitory effect of benzoic acid on SU formation. The conversion of benzoate to hippurate uses the same enzyme system as the conversion of salicylic acid to SU (Levy and Amsel, 1966). Recently, it has been suggested that with long term use, salicylate may induce its own metabolism by increasing the production of SU (Day et al., 1983a; Furst et al., 1977; Rumble et al., 1980). Steady-state salicylic acid concentrations in patients on day 20 of treatment were found to be 48% of those found in the same patients on day 7 (Muller et al., 1975).

Peak concentrations of SAG occur at the same time as peak concentrations of free salicylic acid, but the SPG peak is delayed for 8 hours. After investigating the excretion rate of salicylic acid and the 4 salicylate metabolites, deriving their pharmacokinetic parameters and using computer simulations, the capacity-limited formation of SPG was determined (Tsuchiya and Levy, 1972b) - confirmed by utilising the salicylamide interaction with glucuronide formation (Levy and Procknall, 1968).

GA is formed by the hydroxylation of salicylic acid and is produced in small quantities along with a number of other minor metabolites such as GU and other oxidation products (Wilson et al., 1978). The possibility that GA formation followed Michaelis-Menten kinetics was explored by Boreham and Martin (1969) but this has not been confirmed. Renal excretion of salicylic acid occurs by first-order kinetics and is extremely sensitive to urinary pH (Levy and Leonards, 1971; Smith et al., 1946),

urinary organic acids (Liegler et al., 1966), urinary flow rate (Milne, 1963). The effect of urinary pH on salicylate clearance is most marked at high salicylate concentrations but is still observable at low dosage (Levy and Leonards, 1971). Acid-induced changes in urinary pH (Gibber et al., 1974; Hansten and Hayton, 1980) will cause increases in steady-state plasma salicylate concentration to occur. The renal excretion of SA is reduced by probenecid, which competes for secretion in the proximal tubule (Schachter and Levy, 1958) [see also section 6.5].

4. Pharmacokinetics in Various Age Groups and Disease States

4.1 Children

As well as a reduced albumin concentration, neonates have impaired conjugating mechanisms and thus eliminate maternally-ingested salicylates more slowly than adults (Wolff et al., 1978). Studies in children who had accidentally ingested large amounts of salicylates have shown that V_d varies with the dose; the higher the initial dose, the higher the V_d . This can be explained on the basis of saturation of protein binding sites and changes in plasma pH. This highlights the importance of not using the serum salicylic acid concentration alone in estimating the risk of toxicity in salicylate overdose in children (Done, 1978; Levy and Yaffe, 1974). Pharmacokinetic parameters of salicylate are not influenced by disease states in children (Wilson et al., 1978).

4.2 Elderly

No significant differences exist in the pharmacokinetics of aspirin in the elderly compared with adults. Although one report showed an increase in V_d and a slower rate of elimination of salicylate in the elderly (Cuny et al., 1979), a more extensive single-dose study was unable to demonstrate significant pharmacokinetic differences between the elderly and younger adults (Roberts et al., 1978). Salicylate toxicity may present a specific set of clinical features in the elderly, ranging from

respiratory and encephalopathic features, highlighting the need for monitoring in this age group (Levy, 1982).

4.3 Rheumatoid Arthritis

Serum albumin concentrations are reduced in patients with rheumatoid arthritis, leading to alterations in free salicylate concentrations. There is, however, no significant binding of salicylate to plasma proteins in patients with rheumatoid arthritis (Levy, 1982).

4.4 Renal and Liver Disease

The free fraction of salicylate is increased in patients with renal disease, and may be increased in patients with liver disease. Other pharmacokinetic parameters are unchanged. The higher salicylate concentrations in this group are related to decreased protein binding sites (Lowenthal et al., 1978). The authors used a single dose of 1.73m² and one would expect that if the elimination of SU and free salicylate were increased, toxicity may be reduced.

In a single-dose study, the pharmacokinetics in patients suffering from rheumatoid disease were not different from controls, but unbound salicylate concentrations were significantly higher in patients with disease. This was due to a decrease in protein binding and clearance of salicylate.

5. Clinical Implications of Pharmacokinetic Parameters

The optimal dose of aspirin for platelet adhesiveness and prostacyclin production is small as 160mg results in a significant platelet membrane cyclo-oxygenase inhibition (Burch et al., 1978). Recent studies have demonstrated a significant increase in platelet aggregation following a 650mg

tion and encephalopathy through to hyperventilation, highlighting the importance of careful monitoring in this age group (Vivian and Goldberg, 1982).

4.3 Rheumatoid Arthritis

Serum albumin concentrations are often diminished in patients with rheumatoid arthritis, and hence alterations in free salicylate fraction may occur. There is, however, no evidence that protein binding of salicylate *per se* is altered in patients with rheumatoid arthritis (Wanwimolruk et al., 1982).

4.4 Renal and Liver Disease

The free fraction of salicylic acid has been shown to be increased in patients with renal failure; however other pharmacokinetic features are unchanged. The higher salicylic acid concentrations in this group are related to displacement from protein binding sites (Lowenthal et al., 1974). These authors used a single dose of salicylic acid (500mg/1.73m²) and one would expect that at higher doses of salicylate, toxicity may occur due to impaired elimination of SU and free salicylic acid.

In a single-dose study, aspirin and salicylic acid kinetics in patients suffering from alcoholic liver disease were not different from aged or young controls, but unbound salicylic acid concentrations were significantly higher in those with liver disease. This was due to a decrease in plasma protein binding and clearance of SA (Roberts et al., 1983).

5. Clinical Implications of Pharmacokinetic Properties

The optimal dose of aspirin required to reduce platelet adhesiveness and still maintain vascular prostacyclin production is not known. A dose as small as 160mg results in an 82% acetylation of platelet membrane cyclo-oxygenase at 24 hours (Burch et al., 1978). Recently, Ross-Lee et al. (1982) demonstrated a significant reduction in platelet aggregation following a 650mg dose of enteric-coated

aspirin. Other studies have reported inhibition of platelet aggregation with enteric-coated and sustained-release preparations despite the absence of measurable plasma aspirin concentrations (Brantmark et al., 1982; Siebert et al., 1983).

Evidence suggests that aspirin is a better analgesic than salicylic acid (Lasagna, 1961; Lim, 1966). The analgesia produced by aspirin is dose-dependent, although the response does not parallel serum aspirin concentrations (Levy, 1981). The dose of aspirin required for its antipyretic action is less than that required for analgesia (Wilson et al., 1982).

The generally accepted therapeutic plasma concentration range of salicylate for the treatment of chronic inflammatory disease is 15 to 30mg/100ml (150-300 mg/L or 1-2 mmol/L), requiring daily doses in excess of 3g (Boardman and Hart, 1967; Graham et al., 1977; Miltz et al., 1974; Vesell, 1974). However, this therapeutic plasma concentration range does not appear to have been confirmed with any degree of certainty (Orme, 1982; Smyth and Bravo, 1975).

Side effects of salicylate, in particular tinnitus, are related to the total salicylic acid concentration (Day et al., 1983b) but tinnitus has not been a practical measure of salicylate toxicity in patients with rheumatoid arthritis (Mongan et al., 1973). As the optimum plasma concentration is only slightly below toxic values, care must be taken in the clinical use of salicylates. The rate-limited kinetics of salicylic acid mean that its elimination half-life increases with dose so that a small increase in dose causes more than a proportional increase in the steady-state concentration (Tsuchiya and Levy, 1972a). Paulus et al. (1971) have shown that an increase in the daily salicylate dose from 65 mg/kg to 100 mg/kg results in a 3-fold increase in the plasma salicylic acid concentration. Tsuchiya and Levy (1972a) have shown by computer simulation that it takes only 2 days to reach steady-state salicylate concentrations if a dose of 0.5g is given at a dosing interval of 8 hours. Increasing the dose to 1g with the same dosing interval of 8 hours increases the time taken to reach steady-state concentrations to 7 days, and this doubling of the salicylate dose results in a more than 6-fold increase

in steady-state plasma concentrations.

Calabro et al. (1976) reported that a reduction in the fractional daily dose of aspirin (from 5 to 3 doses) increases the incidence of side effects such as tinnitus. However, Levy and Giacomini (1978) have clearly demonstrated by computer simulation that, providing the daily dose has been correctly chosen, differences in the size of the aspirin dose fraction or the time interval between doses have little effect on steady-state plasma concentrations. Individualisation of dose can be achieved by monitoring plasma concentrations of SA, but a number of other variables affecting salicylate elimination kinetics cannot be readily evaluated from plasma concentration data alone. Nomograms do not adequately take into consideration the pronounced interindividual differences in salicylate elimination, and dose-rate adjustments must therefore be empirically based on information obtained from plasma concentration monitoring and evaluation of the patient's clinical status (Levy and Giacomini, 1978). Therapeutic steady-state salicylic acid concentration during the total 24-hour period would seem to be required for the ideal management of rheumatic disease. There are theoretical reasons which would suggest that the use of enteric-coated or soluble formulations of aspirin given on a 12-hourly schedule, while capable of achieving these therapeutic concentrations, may be associated with toxicity immediately following ingestion. However, in practice this rarely occurs with enteric-coated tablets, and these or sustained-release preparations should be prescribed for long term anti-rheumatic therapy to reduce gastrointestinal blood loss. As an aid to predicting patient response to salicylates in rheumatoid arthritis, Graham et al. (1977) used a 1.2g test dose followed by a serum salicylate concentration measurement 12 hours later. Salicylic acid concentrations greater than 1mg/100ml after the test dose predicted therapeutic concentrations above 15mg/100ml during long term therapy.

A safe approach is to commence therapy with 60 mg/kg/day. Using a 8-hour dosing schedule for 1 week, a blood sample is obtained 1 to 3 hours after a dose and the daily dose is increased to 80

mg/kg/day if the plasma salicylic acid concentration is below 15mg/100ml. Upward dosage adjustment must be made cautiously in small increments and should only be carried out after a thorough clinical and pharmacokinetic assessment of the patient (Levy and Giacomini, 1978). Alternatively, a 12-hour dosage schedule may be employed using sustained-release formulations.

To achieve rapid absorption with early plasma aspirin concentrations, effervescent formulations are appropriate. The addition of metoclopramide can further enhance the rate of absorption and this has been suggested for analgesia in migraine (Rosen et al., 1983).

6. Pharmacokinetic Drug Interactions

6.1 Indomethacin

Since publication of the study by Jerns and Towson (1970) suggesting that concurrent use of indomethacin and aspirin resulted in reduced indomethacin concentrations, conflicting reports have appeared in the literature. However, a clinical study has suggested that no additive therapeutic effect is achieved and there is an increase in side effects with this combination (Brooks et al., 1978).

6.2 Propionic Acid Derivatives

Reductions in the AUC have been reported for ketoprofen (Rubin et al., 1973), naproxen (Rubin et al., 1974), flurbiprofen (Brooks and Khan, 1979) and ibuprofen (Grennan et al., 1979) when administered concurrently with aspirin. However, these actions are probably not of clinical importance.

A more complex interaction exists between ketoprofen and aspirin. Increased plasma concentrations of ketoprofen occur as well as reduced elimination of conjugated metabolites and reabsorption of these conjugates (Williams et al., 1979).

6.3 Diflunisal

The interaction of diflunisal and aspirin is dose dependent, as doses greater than 2.4g of aspirin per day cause significant reductions in diflunisal

concentrations. However, a dose of 100 mg per day, no significant effect was observed (1976).

6.4 Diclofenac

Aspirin has little effect on the plasma concentrations and clearance of diclofenac when administered concurrently (1980). However, the effect on renal function is not clear.

6.5 Phenylbutazone and Sulphinpyrazole

These drugs are contraindicated with aspirin. The combination of aspirin and phenylbutazone has been reported to cause a severe toxic effect (1960; Pascale, 1960). A synergistic effect is also seen with the combination of aspirin and more sensitive drugs such as benecid. Salicylates should be avoided in patients receiving these drugs.

6.6 Methotrexate

Aspirin/methotrexate combination can precipitate methotrexate crystals in the renal tubules, leading to a decrease in the clearance of methotrexate due to decreased renal elimination. This is a therapeutic dose-related effect. An increase in methotrexate toxicity was found in patients receiving this combination (Taylor and Hargrave, 1979).

6.7 Antacids

Aluminium hydroxide and sodium bicarbonate can reduce the urinary pH and thus increase the renal elimination of salicylates. A pH of 8 will increase the renal elimination of salicylates.

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concentrations. However, at doses below 1.2g aspirin per day, no significant effect is seen (Schultz et al., 1976).

6.4 Diclofenac

Aspirin has been shown to reduce the peak concentrations and AUC of diclofenac after intravenous and orally administered doses. Diclofenac Vd and clearance were both increased with aspirin administration (Reiss et al., 1978; Willis et al., 1980). However, the clinical relevance of this interaction is not clear.

6.5 Phenylbutazone, Probenecid and Sulphinpyrazone

These drugs will abolish the uricosuria associated with aspirin doses greater than 2.5 g/day (Oyer, 1960; Pascale, 1955; Tu, 1963). Likewise, the uricosuric effect of probenecid is inhibited by simultaneous low dose aspirin use. A similar effect is also seen with sulphinpyrazone which appears more sensitive to the effect of aspirin than probenecid. Salicylates should be avoided in patients receiving these uricosuric drugs.

6.6 Methotrexate

Aspirin/methotrexate combinations may precipitate methotrexate toxicity (Mandel, 1976). The clearance of methotrexate is reduced 30% by aspirin due to competition between the two drugs for renal elimination (Leigler et al., 1969). Aspirin in therapeutic doses also produces a 20 to 60% decrease in methotrexate protein binding, although this was found to have little clinical significance (Taylor and Halprin, 1977).

6.7 Antacids

Aluminium hydroxide gel, magnesium hydroxide and sodium bicarbonate cause an increase in urinary pH and hence an increased rate of salicylic acid elimination. An increase in urine pH from 5 to 8 will increase the rate of salicylic acid elimin-

ation 20-fold (Smith et al., 1946). However, antacids have no effect on the bioavailability of aspirin (Gibaldi et al., 1974).

6.8 Other Drugs

Many theoretical interactions may occur through displacement of drugs from protein binding sites by aspirin and salicylic acid. Reports indicate that phenytoin (Leonard et al., 1981) and oral hypoglycaemics (Anderson, 1977) fall into this category, but the clinical relevance of these interactions is uncertain. Clinically important interactions may occur with heparin and oral anticoagulants (Hansten, 1975; Prescott, 1969), but these appear to be due mainly to an effect on platelet function rather than on pharmacokinetic parameters.

Drugs that acidify urine, such as ascorbic acid (which may be self-administered in large amounts), will reduce urinary salicylic acid excretion and raise the plasma concentration (Hansten and Hayton, 1980).

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Aspirin and Related Derivatives of Salicylic Acid

Stephen P. Clissold

ADIS Drug Information Services, Auckland

Summary

After almost 90 years of clinical use, aspirin remains one of the world's most extensively used 'over-the-counter' drugs, and it is still recognised as the standard analgesic/antipyretic/anti-inflammatory agent by which newer drugs are assessed. However, its pre-eminent position as the analgesic of choice for mild to moderate pain has been seriously challenged with the introduction of many 'new' non-steroidal non-narcotic analgesic drugs. Indeed, there is convincing scientific evidence that many of the 'newer' non-steroidal drugs such as diflunisal, ibuprofen, flurbiprofen etc. are significantly superior analgesics and, in many cases, have a longer duration of action.

In recent years the salicylates, aspirin in particular, have been the focus of much attention regarding their side effect profiles. At usual dosages for relief of pain and during occasional use, aspirin is well tolerated by the vast majority of patients. Adverse reactions, of which there is a wide spectrum, most frequently accompany anti-inflammatory doses of aspirin, or may be the result of accidental overdosing (particularly in children and the elderly) - probably a reflection of the lay population's acceptability of aspirin's presumed safety. As with other non-steroidal analgesic drugs, gastrointestinal complaints are the most commonly reported side effects.

The existence of many shared clinical, adverse and toxic effects of aspirin and other non-steroidal anti-inflammatory drugs (NSAIDs) is thought to be accounted for by a common mechanism - inhibition of the ubiquitous cyclo-oxygenase enzyme. Thus, suppression of prostaglandin biosynthesis is widely considered to explain the common properties of NSAIDs, although further research is still necessary to clarify some inconsistencies and to complete our understanding of the processes involved.

Aspirin and salicylates have been reported to have a wide range of drug interactions but only relatively few seem to be clinically important. Many of the interactions are pharmacokinetic in nature. Drugs considered to produce the most significant interactions with salicylates include anticoagulants and thrombolytic agents, uricosuric agents, corticosteroids, methotrexate and sulphonylurea hypoglycaemic agents.

Numerous historical accounts of the use of plant materials containing salicin, extraction of the active material salicylic acid, and the synthesis of active derivatives, especially acetylsalicylic acid (aspirin), have been published (e.g. Hanks, 1982;

Miller, 1982; Starmer, 1983). Aspirin was first administered clinically at the turn of the century and now, almost 90 years later, it still remains the world's most extensively used 'over-the-counter' drug. In the United States alone, it has been esti-

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estimated that approximately 20,000 million aspirin tablets are consumed per annum and this represents about 100 tablets/year for every man, woman and child (Taylor, 1980-81). The fact that aspirin has remained so popular for so long highlights its acceptability to the general public and, presumably, is a good indication of its therapeutic usefulness for treating minor aches and pains.

Although aspirin is generally considered safe by most people, it is certainly not innocuous; side effects, especially gastrointestinal disturbances, do occur (see Ivey, this issue). Additionally, aspirin is a common cause of analgesic poisoning in both adults and children. Accidental overdosing, particularly in the elderly and in children, is probably a reflection of the drug's popularity and wide availability, and the lay population's acceptability of its efficacy and presumed safety.

It is against this background that the search for safer and more effective salicylates has been carried out. This review will focus on the pharmacodynamic properties (analgesic and anti-inflammatory activity), pharmacokinetic characteristics

and therapeutic efficacy (including adverse effects and drug interactions) of aspirin, the standard non-narcotic analgesic by which newer salicylates are assessed. Where appropriate, some perspective of how other derivatives of salicylic acid (such as diflunisal, fendosal, fosfosal and salsalate) compare with aspirin will be presented. (For structural formulae of these compounds, see fig. 1.)

1. Pharmacodynamic Properties

1.1 Mechanism of Action

Although aspirin is the most widely used non-narcotic analgesic, it was not until the early 1970s that a major discovery was made regarding its mechanism of action. The demonstration in 1971 that aspirin inhibited the enzymatic production of prostaglandins initiated intense investigation of the physiological role of these autacoids (Ferreira et al., 1971; Smith and Willis, 1971; Vane, 1971). It is now widely accepted that many of the therapeutic and unwanted effects of aspirin (and other salicylates) can be attributed to a reduction in the bio-

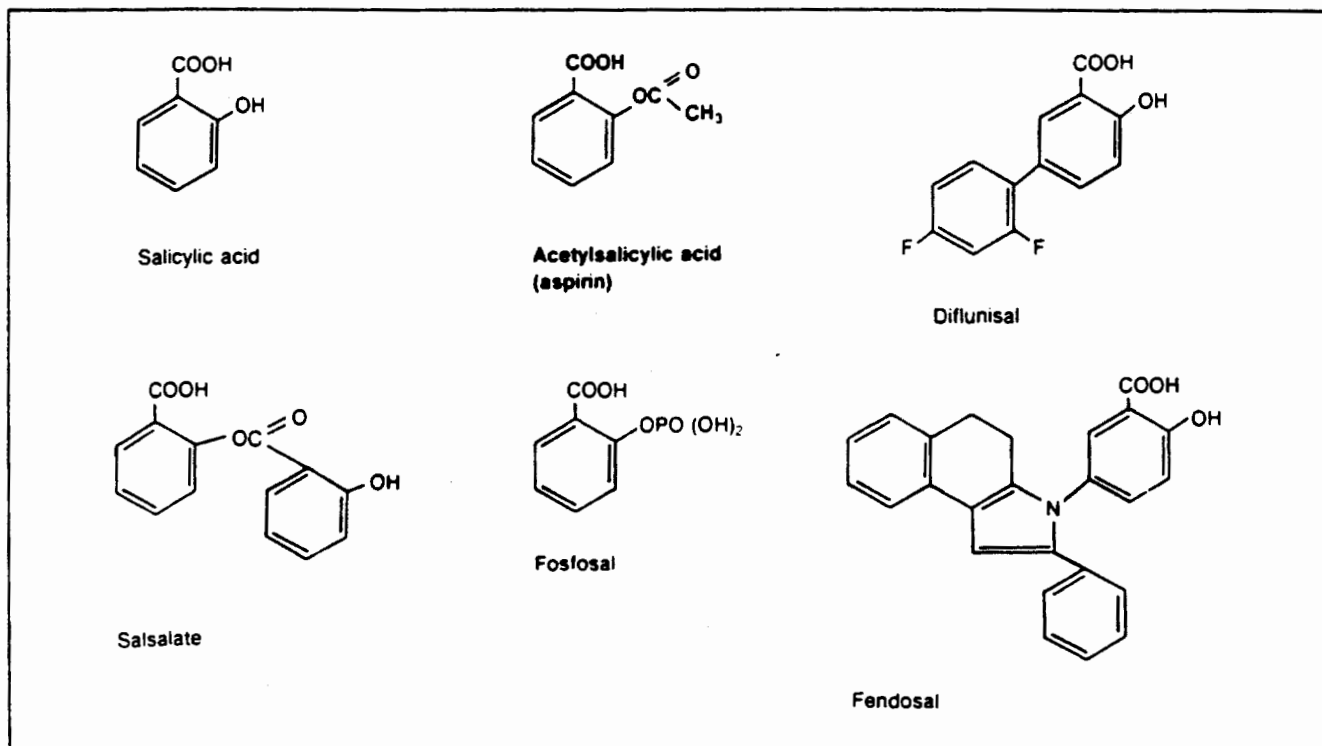


Fig. 1. Structural formulae of salicylic acid, aspirin and other derivatives of salicylic acid with analgesic properties.

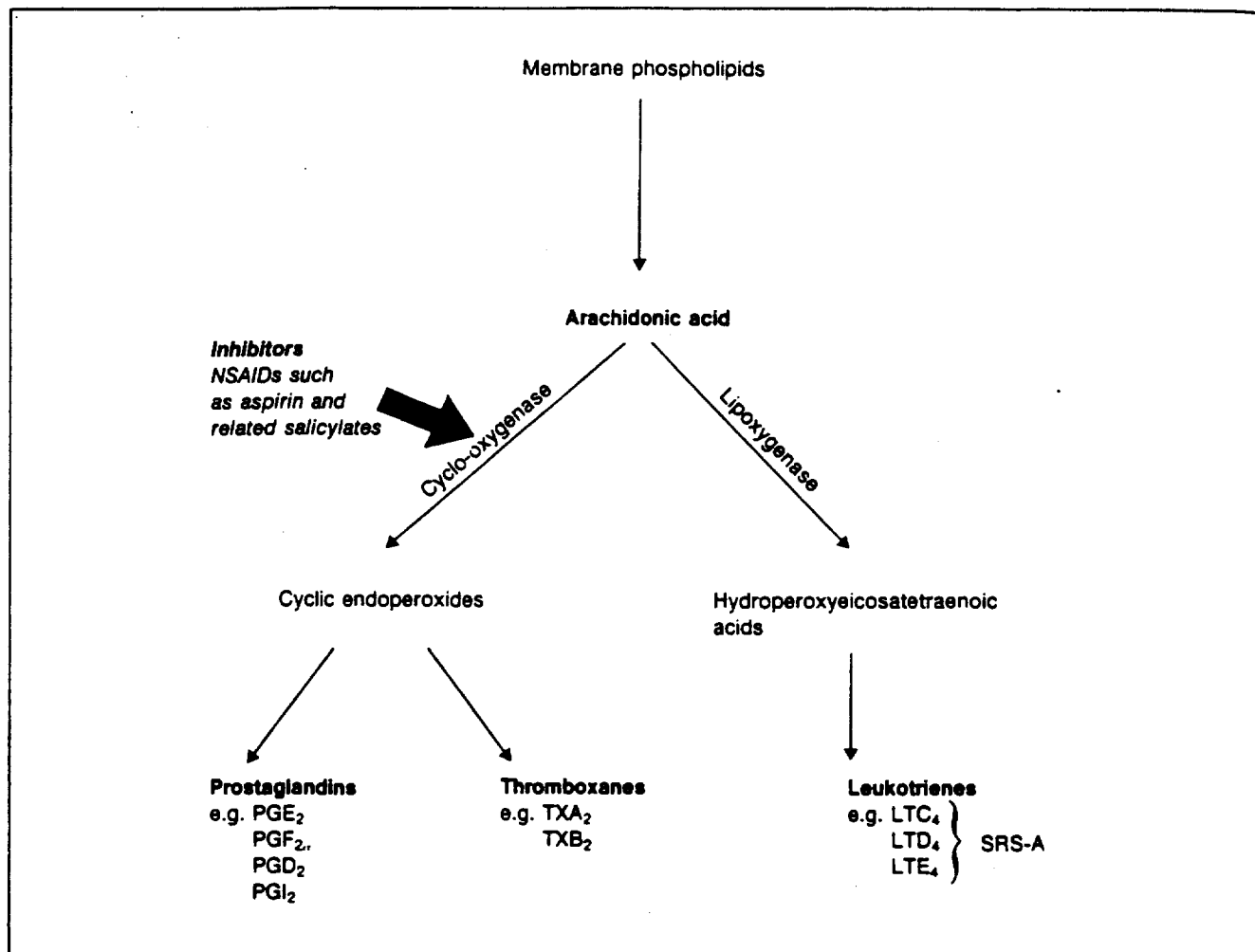


Fig. 2. Simplified schematic representation of cyclo-oxygenase and lipoxygenase pathways leading to the production of prostaglandins, thromboxanes and leukotrienes from arachidonic acid (adapted from Brune and Lanz, 1984; Farah and Rosenberg, 1980; Kay, 1983; Moncada et al., 1980).

synthesis of prostaglandins and related autacoids from arachidonic acid. In the multiplicity of steps leading to the production of prostaglandins, NSAIDs such as aspirin prevent the synthesis of cyclic endoperoxides by the prostaglandin synthetase enzyme cyclo-oxygenase (fig. 2).

Certain intermediates and end products of the arachidonic acid cascade, in combination with other local mediators such as bradykinin, histamine and 5-hydroxytryptamine, may cause erythema, oedema, pain, etc. associated with the inflammatory response. Because the prostaglandins are among the most prevalent of autacoids, inhibition of cyclo-oxygenase probably explains, at least in part, the anti-inflammatory activity of aspirin as well as its

actions on gastric mucosa, platelets, kidney, uterus, etc. (Vane, 1975).

In vivo aspirin is rapidly hydrolysed to salicylic acid and acetate and many of its pharmacodynamic actions are attributable to the salicylate moiety. However, hydrolysis is not a prerequisite for pharmacological activity and aspirin itself has some unique properties (for a detailed review see Flower et al., 1980). Interestingly, aspirin inhibits cyclo-oxygenase by acetylation, whereas salicylic acid, which has no acetylating capacity, has little effect on the enzyme *in vitro*. Nevertheless, the 2 drugs produce comparable inhibition of prostaglandin biosynthesis *in vivo*. These findings not only suggest a different mechanism of action for aspirin

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compared with the salicylate moiety; they also indicate that aspirin probably possesses a dual means of inhibiting cyclo-oxygenase. Early studies found aspirin to be a superior analgesic to sodium salicylate (Lasagna, 1961; Lim, 1966) and presumably this implies that acetylation of cyclo-oxygenase is an important mechanism for its analgesic properties. Recent evidence from Seymour et al. (1984) appears to support this contention, although further study is obviously needed to clarify how other non-acetyl-containing non-steroidal drugs produce their analgesic effects.

Inhibition of prostaglandin production does not seem to explain all of the pharmacodynamic actions of aspirin and additional mechanisms have been postulated (for reviews see Brune and Lanza, 1984; Hanks, 1982). It has been speculated that high doses of aspirin may inhibit hydroperoxy fatty acid peroxidase in the lipoxygenase pathway of arachidonic acid metabolism and this may contribute to some of the effects of aspirin in man (McDonald-Gibson et al., 1984). Of course, unknown peripheral and/or central mechanisms of action may also be involved. Further research is clearly needed to explain some of the inconsistencies in the literature, to clarify the exact mechanism of action of aspirin and other derivatives of salicylic acid, and to fully elucidate our understanding of the involvement of the end products of the arachidonic acid cascade in various physiological systems.

1.2 Analgesic Effects

Aspirin and related drugs can alleviate pain of mild to moderate severity. These effects appear to be mediated through peripheral and central mechanisms, although the work of Lim et al. (1964) suggests that aspirin acts mainly peripherally (see also Bowman and Rand, 1980; Flower et al., 1980).

Prostaglandins seem to sensitise peripheral pain receptors to mechanical or chemical (bradykinin, histamine, etc.) stimulation at a local level. Most data are consistent with the theory that aspirin and other cyclo-oxygenase inhibitors avert the sensitisation of peripheral nociceptors to such stimulation by preventing production and consequently release

of the intermediates and/or end products of the arachidonic acid cascade. These drugs do not affect the sensitisation or pain caused by the direct action of prostaglandins, which is in agreement with this hypothesis. Additionally, cyclo-oxygenase inhibitors are most effective against dull, throbbing pain associated with inflammation (where prostaglandins apparently sensitise the nerve endings) and are less effective against sharp, stabbing pain caused by direct stimulation of sensory nerves.

In addition to these peripheral actions, direct effects of salicylates within the central nervous system (CNS) have also been described (Dubas and Parker, 1971). The mechanisms involved in such central activity have not been clearly defined, although inhibition of prostaglandin production within the CNS remains a possibility. Since analgesic doses of aspirin do not cause mental disturbances, hypnosis, or striking changes of mood, relieve pain without affecting other sensory modalities, and do not modify arousal mechanisms involving the brain stem reticular formation, it seems likely that any central mechanism is probably subcortical, perhaps at the level of the hypothalamus. The paucity of such CNS effects also suggests that the analgesic activity of salicylates is mediated largely through their peripheral actions.

1.3 Anti-Inflammatory Effects

This review was intended to cover the analgesic characteristics of the salicylates, but in some ways it is not possible to divorce these actions from the anti-inflammatory effects and, consequently, a brief overview of the anti-inflammatory properties of aspirin is needed. (For reviews see Bowman and Rand, 1980; Flower et al., 1980.)

Aspirin has been used since about 1900 for the treatment of inflammatory diseases such as rheumatoid arthritis, and it was not until very recently that other NSAIDs challenged its pre-eminence as an antirheumatic drug. At present it does not seem that any of the newer non-steroidal drugs are more effective anti-inflammatory agents, but at therapeutic dosages many of them are generally better tolerated (see p. 27).

Inflammation is an extremely complex defensive reaction to injury, which is characterised by erythema, oedema, tenderness and pain. As a result of cell damage, chemical mediators such as histamine, bradykinin, 5-hydroxytryptamine, slow-reacting substance of anaphylaxis (SRS-A), chemotactic factors, prostaglandins, etc. are liberated locally. As the inflammatory response progresses, the vascular endothelium becomes swollen and eventually blood elements leak into the interstitial spaces. Phagocytic cells (including leucocytes) migrate to the damaged area and probably contribute to the defensive reaction by releasing lytic enzymes. Any pain associated with inflammation is probably due to stimulation of nerve endings by one or more of the chemical mediators released during the host's response to injury. This somewhat simplistic outline of the acute inflammatory response to tissue damage serves to highlight the difficulties involved in evaluating the mechanism of action of aspirin during such a complex series of events. Aspirin and other NSAIDs are effective in modifying the inflammatory response in a variety of rheumatic diseases, but they are not curative, in that they do not stop the degenerative process.

Prostaglandins are released during the inflammatory response and they seem to be important mediators of the defensive reaction. Hence, the ability of salicylates to inhibit prostaglandin synthesis could account for their anti-inflammatory properties. However, as noted by Sause et al. (1982), various other mechanisms have been proposed such as interference with cellular metabolism, interference with the release of inflammatory mediators from plasma proteins, interference of sodium and potassium ion transfer across cell membranes, inhibition of the actions of chemical mediators other than prostaglandins, and stabilisation of lysosomes. Furthermore, the discovery of the lipooxygenase pathway and the production of leukotrienes adds another avenue which may contribute to the underlying process of inflammation. It is generally agreed, however, that the anti-inflammatory effects of NSAIDs are positively correlated with their anticyclo-oxygenase activity (Vane, 1973). Further studies are clearly necessary

to elucidate the exact mechanisms by which salicylates produce their anti-inflammatory effects.

2. Pharmacokinetic Properties

Aspirin and some other derivatives of salicylic acid (e.g. choline salicylate, choline magnesium trisalicylate, magnesium salicylate, salsalate) are hydrolysed in the body to salicylic acid. Irrespective of the parent source of salicylate, once it is absorbed and hydrolysed the pharmacokinetic characteristics are essentially those of salicylic acid. It follows that blood and urinary data for salicylate are more meaningful than blood or urine concentrations of the parent drug (Dromgoole et al., 1981). Only in the last two decades have the complex pharmacokinetic properties of the salicylates been fully appreciated, and some aspects are still unresolved (Levy, 1981). Other derivatives of salicylic acid (notably diflunisal) are not hydrolysed to salicylate and will be discussed briefly where appropriate.

There is growing evidence that the pharmacokinetic behaviour of NSAIDs has a major impact on their therapeutic efficacy and tolerability (for recent reviews see Brune and Lanz, 1984, 1985). Indeed, it has been shown that these drugs achieve particularly high concentrations in those compartments in which they have beneficial and/or adverse effects. Consequently, an understanding of the pharmacokinetic properties of aspirin and related salicylic acid derivatives is of prime importance to our interpretation of the clinical attributes of this class of drugs.

2.1 Absorption

Orally administered aspirin is rapidly and usually completely absorbed from the gastrointestinal tract (both as unchanged drug and hydrolysed salicylate) [Dromgoole et al., 1981]. Absorption occurs by passive diffusion of un-ionised lipophilic molecules, partly from the stomach but mainly from the upper small intestine. Many factors are known to affect the rate of absorption (table I) and, of these, drug formulation has the major influence since it

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Derivatives of salicylic acid (e.g., sodium salicylate, magnesium salicylate) are weak acids. Irrespective of the route of administration, once it is absorbed, the pharmacokinetics of salicylic acid are similar to those of salicylate (Rowland et al., 1981). The complex formation of salicylates with proteins has not been fully elucidated and is still unresolved. The hydrolysis of salicylic acid to salicylic acid is a reversible process.

The pharmacokinetics of salicylic acid have a major impact on its bioavailability (for review, see Rowland, 1984, 1985). The drugs achieve a steady-state concentration in the blood compartment and/or in the tissues. A better understanding of the pharmacokinetics of aspirin and related compounds is of prime importance for clinical attributes.

Aspirin is rapidly and usually absorbed in the gastrointestinal tract. Hydrolysis of salicylic acid occurs in the stomach and in the small intestine. The hydrolysis is mainly from the ester bond. The known mechanism of these processes is of importance since it

controls the dissolution of aspirin and this has been shown to be the rate-limiting step for the absorption of solid tablets (Levy and Hollister, 1965). Overall, effervescent and soluble tablet preparations are most rapidly absorbed (15 to 40 minutes), followed by uncoated or film-coated tablets (25 to 60 minutes), and finally by enteric-coated (240 to 360 minutes) and extended-release formulations (60 to 120 minutes). The times in parentheses represent the approximate times to achieve mean peak aspirin concentrations; however, plasma aspirin concentrations decline rapidly as plasma salicylic acid concentrations increase. Thus, the corresponding times to achieve peak salicylic acid concentrations are 30 to 60 minutes for soluble or effervescent tablets, 45 to 120 minutes for uncoated or film-coated tablets, 4 to 12 hours for extended-release tablets, and 8 to 14 hours for enteric-coated tablets.

After oral administration of an aqueous solution, the absorption of aspirin was found to follow first-order kinetics (Rowland et al., 1972). In this study there was a wide variation in absorption half-life (4.5 to 16 minutes) and approximately 70% of the administered dose reached the systemic circulation unchanged. The remaining 30% was thought to be hydrolysed during absorption by esterases within the gut wall, plasma or liver.

Gastrointestinal pH has a major influence on the rate of absorption of aspirin by two different

mechanisms. Firstly, low pH in the stomach provides optimum conditions for the absorption of undissociated aspirin molecules, although this is probably not important for drug preparations which are given in solution (effervescent and soluble tablets). Secondly, as pH rises (in the small intestine) the dissolution rate for aspirin tablets increases, and is maximal at pH 8 (for a recent review see Needs and Brooks, 1985). Some formulations of aspirin contain various buffers, but the effects of these on drug absorption are both variable and conflicting. Certainly, it now seems unlikely that buffered aspirin tablets cause less gastric irritation than uncoated plain aspirin tablets, as was previously thought (see Ivey, this issue).

Salsalate is completely absorbed from the gastrointestinal tract, although the relative amount reaching the circulation as unchanged drug has not been reported. Mean peak salsalate concentrations have been noted after approximately 1.5 hours; mean peak salicylic acid concentrations occurred within 2 to 4 hours. As for most derivatives of salicylic acid, food delays the absorption of salsalate and also reduces the mean peak plasma concentration.

Diffunisal is rapidly and completely absorbed after oral administration, generally achieving peak plasma concentrations within 2 to 3 hours. In fasted subjects the bioavailability of diffunisal is significantly decreased by concomitant administration of antacids containing aluminium hydroxide (Brogden et al., 1980). Because diffunisal has a long half-life, steady-state plasma concentrations are not attained for 3 to 4 days with low doses (125mg) and for 7 to 9 days with higher doses (500mg) administered twice daily.

2.2 Distribution

Once absorbed, aspirin is rapidly hydrolysed to salicylic acid with a half-life of only 15 to 20 minutes (Rowland and Riegelman, 1968). Thus, from this stage onwards the pharmacokinetics of aspirin, and other salicylates hydrolysed to salicylic acid, are predominantly dependent upon the salicylate moiety (Needs and Brooks, 1985) [see fig. 3].

Table 1. Some important factors affecting the rate of salicylate absorption (after Needs and Brooks, 1985)

Drug formulation
pH of stomach contents
Rate of gastric emptying
Volume of food
Concurrent administration of other drugs
Nervous state
Posture
Exercise
Disease states associated with altered gastrointestinal transit time

Salicylic acid is normally highly protein bound (80 to 90%) at therapeutic plasma concentrations and this probably accounts for the low reported values for the apparent volume of distribution, which range between 9.6 and 12.7L in adults and between 0.12 and 0.14 L/kg in children (Graham et al., 1977; Wilson et al., 1982). Despite such low values for the apparent volume of distribution, salicylic acid rapidly distributes throughout extracellular fluid and into most tissues. Distribution appears to increase with increasing doses and this may be due, at least in part, to decreased protein binding at higher plasma salicylate concentrations. Salicylic acid protein binding is closely dependent upon serum albumin concentrations; if these decrease (e.g. during pregnancy and in patients with renal disease), the bound salicylate fraction decreases and the amount of free drug increases proportionally.

It has been suggested that the binding of salicylic acid to the albumin molecule occurs at 2 primary and a number of secondary binding sites (Borgå et al., 1976).

Whereas salicylic acid is largely bound to plasma albumin, aspirin itself binds only poorly to plasma proteins, although it has been shown to acetylate plasma albumin as well as some enzymes such as cyclo-oxygenase. This latter effect is probably a major mechanism by which aspirin produces its analgesic and anti-inflammatory actions (see sections 1.1, 1.2 and 1.3).

After absorption, salicylate penetrates into synovial fluid, peritoneal fluid, saliva and milk, but not into gastric juice and only slightly into bile, sweat and inflammatory exudates. It readily crosses the placental barrier. Distribution of salicylate occurs by pH-dependent passive diffusion, which

Absorption

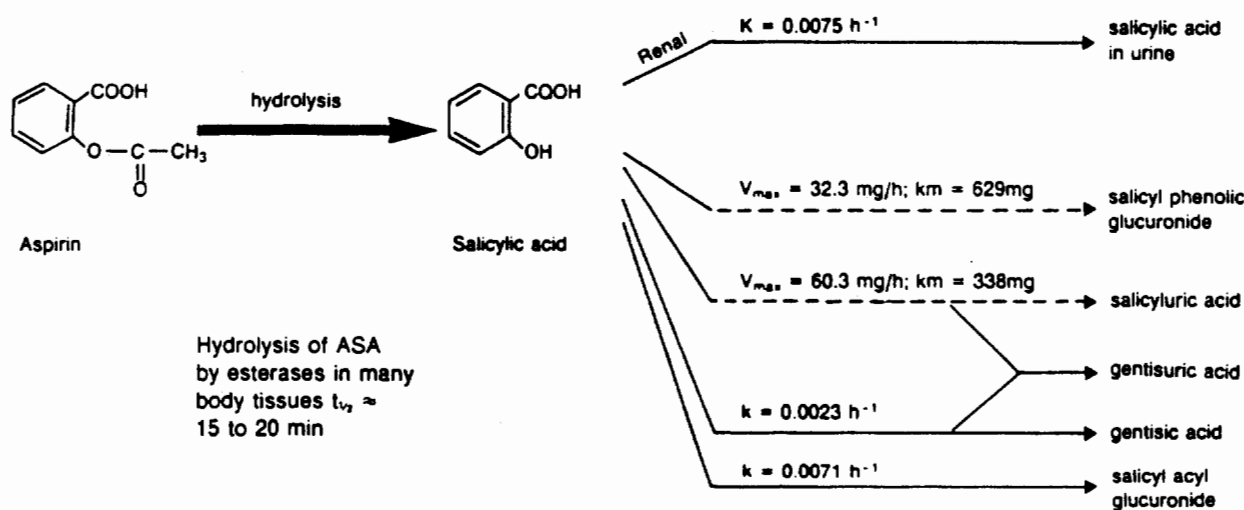
70% ASA absorbed unchanged
 T_{max} of sol. ASA \approx 25 min
 T_{max} of enteric-coated ASA \approx 4 to 6 hours

Disposition

Highly protein bound
 \approx 80-90%
 Vol. \approx 9 to 13L

Elimination

biexponential $t_{1/2\alpha}$ \approx 13-19 min
 SA removed by 5 parallel competing pathways: 1 renal and 4 metabolic



Excretion

varies depending on dose and urinary pH -
 at low doses approximately 90% is excreted
 as salicyluric acid and salicyl phenolic glucuronide.

Fig. 3. Some important pharmacokinetic characteristics of aspirin (ASA) and salicylic acid (SA) in man. Solid lines represent first-order processes and Michaelis-Menten pathways (after Levy and Tsuchiya, 1972; Needs and Brooks, 1985).

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binding sites

limits its ability to cross the blood-brain barrier (Flower et al., 1980).

Diffenol is 98 to 99% plasma protein bound and has a relatively low apparent volume of distribution of about 8L in patients with normal renal function (Brogden et al., 1980). The volume of distribution is significantly increased to almost 17L in patients with terminal renal insufficiency. In lactating women, the milk diffenol concentration is 2 to 7% of that in plasma.

2.3 Elimination

As emphasised earlier, aspirin is rapidly metabolised to salicylic acid; the acetyl component is generally found in the gastric mucosa or is excreted as carbon dioxide after passing through the Krebs cycle (Rainsford et al., 1983). Following intravenous administration, the elimination kinetics of aspirin are best described by a biexponential equation with a terminal half-life of 13 to 19 minutes (Rowland and Riegelman, 1968).

The elimination of salicylic acid is far more complex and it is removed from the body by 5 parallel and competing pathways; 1 renal and 4 metabolic (fig. 3). The 3 main metabolites are salicyluric acid (glycine conjugate), salicyl phenolic glucuronide and salicyl acyl glucuronide. Small amounts of salicylate undergo oxidation to gentisic acid; gentisuric acid may be formed from it by glycine conjugation or from salicyluric acid by microsomal oxidation (Wilson et al., 1978). Because the 2 major metabolites of salicylic acid (salicyluric acid and salicyl phenolic glucuronide) are produced via saturable Michaelis-Menten pathways, the elimination kinetics are highly dose dependent. The other routes of elimination exhibit linear first-order kinetics (Levy, 1965, 1971, 1979) [fig. 3].

Biotransformation of salicylate occurs in many tissues but particularly in the endoplasmic reticulum and mitochondria of the liver (Flower et al., 1980). Some salicylic acid is excreted unchanged via the kidney and the various metabolites are also eliminated renally. The relative amounts of salicylic acid and its conjugates excreted in the urine vary widely, being dependent upon the dose ad-

ministered and urinary pH. Notably, at high doses the excretion of unchanged salicylic acid, salicyl acyl glucuronide and gentisic acid generally increases as a result of the saturable nature of the pathways forming salicyluric acid and salicyl phenolic glucuronide (at small doses of aspirin, 300mg or less, about 90% is excreted via these saturable routes). As urinary pH is increased from acidic to alkaline, the urinary excretion rate for free salicylic acid is markedly elevated, and the fraction of a single dose eliminated in the urine as unchanged drug may increase from as low as 5% to as high as 85%.

Reported values for the plasma half-life of salicylic acid are also dose dependent. It has been shown that at low doses (e.g. 325mg aspirin) salicylate elimination is first order with a half-life of about 2 to 3 hours. At higher doses, elimination of salicylate is limited by the ability of the liver to form salicyluric acid and salicyl phenolic glucuronide, and plasma half-life may increase up to 30 hours or more.

Salsalate is rapidly and extensively hydrolysed to 2 molecules of salicylate in the body (about 7 to 13% of a single oral dose is conjugated with glucuronide before hydrolysis). The drug is primarily excreted as salicylate metabolites by the kidneys: small amounts are eliminated as unchanged salsalate, some as a glucuronide metabolite, and most via the salicylic acid pathway. The plasma elimination half-life of salsalate is about 1 hour.

Unlike the drugs mentioned so far, diffenol is not metabolised to salicylic acid and its elimination is almost entirely dependent on glucuronidation. About 80 to 95% of an oral dose of diffenol is excreted in the urine in 72 to 96 hours with a terminal elimination half-life of about 10 hours. The half-life is considerably increased in patients with renal insufficiency (up to 115 hours when creatinine clearance is less than 0.12 L/hour) [Brogden et al., 1980].

3. Analgesic Efficacy

Aspirin and other salicylates are weaker analgesics than most narcotic drugs and they are most effective in relieving mild to moderate pain such

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as toothache, headache, arthralgia, dysmenorrhoea, discomfort (and fever) associated with common colds, and a wide range of muscular aches and pains. Salicylates are also useful in suppressing other forms of mild to moderate pain, such as postsurgical pain (dental, orthopaedic, general), postpartum/episiotomy pain and chronic pain of visceral origin in cancer patients. When used to treat pain associated with inflammatory disorders (e.g. rheumatoid arthritis, osteoarthritis and other rheumatic conditions) appropriately employed dosages of salicylates, particularly aspirin, compare favourably with other NSAIDs. This action of salicylates is dependent on their anti-inflammatory properties and will not be considered in any detail in this review. The aim is to evaluate the analgesic efficacy of this class of drugs and this has been most extensively studied in acute pain conditions, generally of a self-limiting nature (postsurgical pain, episiotomy pain, dental pain, etc.), in single-dose comparative clinical trials.

3.1 Acute Pain

Aspirin has been compared with placebo, many other non-narcotic analgesics (including other salicylates) and some centrally acting analgesics in a variety of acute pain conditions (for detailed reviews see Cooper, 1981, 1983; Seymour, 1983).

3.1.1 Comparisons with Placebo

In a recent review, Seymour (1983) reported that in almost all clinical trials in patients with dental pain following third molar surgery, aspirin, as might be expected, was significantly superior to placebo. In an extensive study, von Graffenried et al. (1980) found that in 5 separate placebo-controlled clinical trials aspirin 1g produced significantly ($p < 0.01$) better pain relief than placebo in 326 subjects after removal of impacted lower molars. Using 2 different doses of aspirin, Seymour and Rawlins (1982) demonstrated that a 600mg dose was only significantly superior to placebo in relieving postsurgical dental pain 45 minutes after administration. Aspirin 1200mg, on the other hand, produced significantly better pain relief at all time points be-

tween 45 and 240 minutes. Only isolated trials have failed to identify a significant advantage for aspirin compared with placebo, and these may not have employed methods sufficiently sensitive to record pain differences. In many other clinical studies in which a placebo period has been included, aspirin nearly always produced significantly greater pain relief than placebo (Cooper, 1983).

The analgesic efficacy of salsalate in acute pain conditions has not been extensively evaluated, although it is generally considered to be as effective as aspirin in chronic pain conditions associated with rheumatic diseases. In a placebo-controlled dose-comparative study, Forbes et al. (1982b) demonstrated that diflunisal at doses between 250 and 1000mg produced pain relief significantly superior to that provided by placebo and aspirin 650mg in 201 patients with postoperative pain after oral surgery (see fig. 4). The same group found that fentanyl, a recently developed salicylic acid derivative, was also significantly better than placebo in providing pain relief after dental surgery (Forbes et al., 1984).

3.1.2 Comparative Efficacy of Some Derivatives of Salicylic Acid

Aspirin appears to be a better analgesic than sodium salicylate (Mehlich, 1983; see section 1.1). A double-blind crossover trial in patients with pain resulting from dental surgery reported no difference in analgesic efficacy between sodium salicylate 537 and 1074mg and placebo (Seymour et al., 1984). However, the number of trials comparing aspirin and sodium salicylate are very few, and additional supporting data for this statement are still needed. Indeed, there is limited information regarding the comparative analgesic efficacy of most salicylates, although 'newer' derivatives have usually been compared with aspirin.

Diflunisal has been evaluated in a number of pain states and has been compared with a number of non-narcotic analgesics, particularly aspirin (for detailed reviews see Brogden et al., 1980; Cooper, 1983). In postoperative dental pain patients, diflunisal 250 to 1000mg was found to have a higher peak effect and a substantially longer duration of

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action than aspirin 650mg (Forbes et al., 1982b) [fig. 4]. Comparisons between diflunisal and aspirin in patients with other forms of postoperative pain (orthopaedic, episiotomy, meniscectomy, general) have generally shown that the 2 drugs have similar peak effects but that diflunisal 500 to 1000mg has an 8 to 12 hour duration of action. This reflects the relatively long plasma half-life for diflunisal (about 10 hours; see section 2.3), which permits twice daily administration.

A similar analgesic profile has recently been reported for fendosal. Compared with aspirin 650mg, fendosal 200mg produced a slower onset of action (3 hours vs 1 hour), had a comparable peak analgesic effect, and had a significantly longer duration of analgesia (8 hours vs 2 hours) in 109 patients with pain after dental surgery (Forbes et al., 1984).

3.1.3 Comparisons with Paracetamol (Acetaminophen)

Aspirin and paracetamol have been compared in a variety of painful conditions such as postoperative pain resulting from oral surgery and dental extractions, episiotomy pain and pain associated

with malignancy. In reviewing clinical trials up to 1981, Cooper (1981) concluded that aspirin and paracetamol are equianalgesic and, on a weight for weight basis, equipotent in most types of pain. A similar conclusion was reached by Mehlisch (1983). The 2 drugs have similar dose-response and time-effect curves (Cooper, 1983) [fig. 5].

Peak analgesic effects of diflunisal 500 and 1000mg were found to be significantly greater than that of paracetamol 600mg and comparable with that of a combination of paracetamol 600mg and codeine 60mg (Forbes et al., 1982a). Similarly, Melzack et al. (1983) demonstrated that diflunisal 1000mg produced a significantly greater reduction in pain than paracetamol 650mg from hours 5 to 12. Other studies have found that diflunisal 500mg is a superior analgesic to paracetamol 500 or 1000mg (Quiding et al., 1985); diflunisal 250mg and a combination of paracetamol 500mg, codeine phosphate 8mg and caffeine 30mg were equally efficacious (White and Strunin, 1982); and diflunisal 500mg twice daily produced better relief from night pain and pain associated with passive movement and tenderness than a combination of paracetamol and dextropropoxyphene (Rao and Sharma, 1982). In these studies diflunisal had a significantly more prolonged duration of action, although its onset may be slightly slower.

3.1.4 Comparisons with Other NSAIDs

Cooper (1983) has recently reviewed the available data concerning the analgesic efficacy of NSAIDs including the salicylates and paracetamol. This extensive review focused on the use of these drugs in acute pain conditions, but mainly pain associated with oral surgery. In general terms the results clearly demonstrated the greater analgesic effects of newer NSAIDs such as ibuprofen, indoprofen, suprofen, ketoprofen, flurbiprofen, zomepirac, etc. Advantages such as quicker onset of action or a more sustained duration of action have also been reported for some of these drugs. The author suggests that the newer non-steroidal drugs appear to fill a void between mild non-narcotic analgesics and potent injectable narcotic analgesics. An additional advantage of the newer NSAIDs

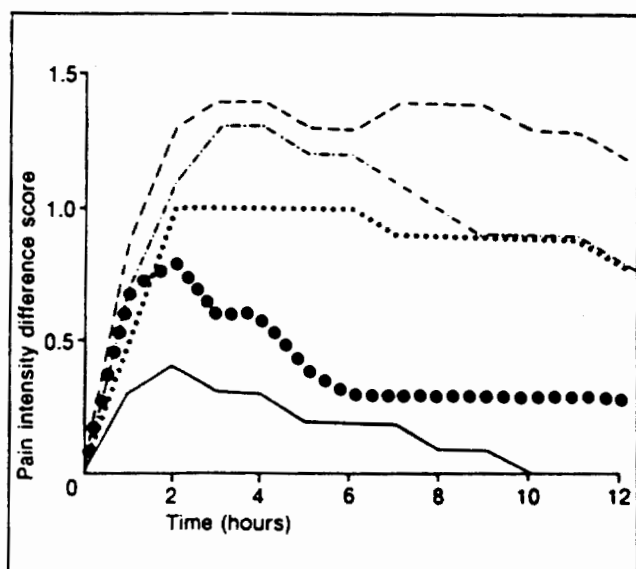


Fig. 4. Change in mean pain intensity difference score with time following administration of placebo (—, n = 38), aspirin 650mg (···, n = 42), diflunisal 250mg (---, n = 39), diflunisal 500mg (· · · · ·, n = 41), and diflunisal 1000mg (— · — · —, n = 41) in 201 patients with postoperative pain following oral surgery (adapted from Forbes et al., 1982b).

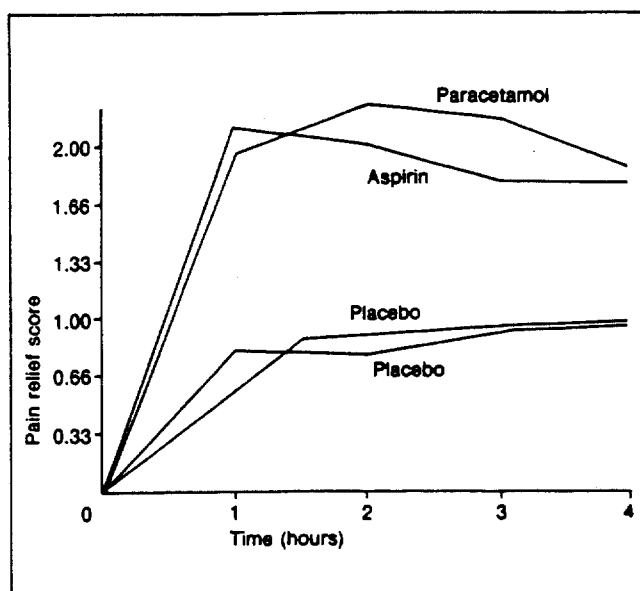


Fig. 5. Composite time-effect curve from 2 separate clinical studies comparing aspirin 650mg with placebo, and paracetamol 650mg with placebo in patients with pain following oral surgery (from Cooper, 1981, with permission).

is that they almost all have more favourable side effect profiles than aspirin. However, when aspirin has been contraindicated these newer drugs are also contraindicated, or they should be used with extreme caution (see Szczeklik, this issue). The analgesic properties of NSAIDs are discussed more fully by Brogden (this issue, p. 27).

Ibuprofen was the first propionic acid derivative with analgesic properties to be marketed and it has only recently received approval for over-the-counter supply. It will be of great interest to see the extent to which it makes in-roads into the tight hold that paracetamol and aspirin have had on the mild to moderate analgesic market.

3.1.5 Comparisons with Centrally Acting Mild to Moderate Analgesics

Aspirin has been compared with a small number of narcotic analgesics such as codeine, pentazocine and dextropropoxyphene, which are generally used to treat mild to moderate pain (for a recent review see Seymour, 1983). Single dose studies in patients with postoperative dental pain demonstrated that aspirin is a superior analgesic to these

3 drugs. Cooper and Beaver (1976) showed that aspirin 650mg gave considerably better pain relief than codeine 60mg and the combination of these 2 drugs was even more effective.

Diffunisal 500mg was found to be superior to low doses of codeine in patients with pain resulting from the removal of impacted mandibular wisdom teeth and as effective as pentazocine 50mg in patients with mild to moderate pain resulting from cancer.

3.2 Visceral Pain Associated with Cancer

Malignant diseases may cause pain in a variety of ways. Usually direct local pressure from a tumour on a nerve, sensitive tissues, viscera or bone is the main mechanism; indirect effects may also be responsible (Woods, 1983). Salicylates provide relief from mild forms of pain, but usually the pain is severe and stronger analgesics are required. In a double-blind crossover study, various analgesics were compared in 57 patients with mild to moderate pain resulting from an unresectable malignancy (Moertel et al., 1972). Aspirin 650mg produced better pain relief in a greater number of patients than most of the other mild to moderate analgesics tested.

4. Side Effects

Occasional use of usual dosages of aspirin for analgesic or antipyretic purposes generally causes few adverse effects. However, when large doses are administered or when it is given for sustained periods, the incidence of side effects increases; these reactions are probably related to the limited capacity of the metabolic pathways responsible for the elimination of aspirin (see section 2.3).

In recent years, salicylates (particularly aspirin) have been the subject of a great deal of attention with regard to their side effect profiles. Considering aspirin's wide usage, it is perhaps not surprising that it has been reported to cause a multiplicity of adverse reactions and to affect many of the organs of the body. However, as noted by Jick (1981), even without collecting formal data, it does seem as

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though serious side effects with aspirin are rare; if this were not the case, the extensive use of the drug would result in a continuous epidemic of severe adverse toxic reactions.

Some of the more recently developed salicylates such as diflunisal and salsalate seem to have improved side effect profiles compared with aspirin; these drugs may be more reasonable choices for the longer term treatment of chronic diseases such as rheumatoid arthritis. However, if aspirin is contraindicated these drugs are probably also contraindicated or, at best, they should be introduced with caution.

4.1 Gastrointestinal Effects

Gastrointestinal complaints are the most frequently reported side effects associated with the administration of NSAIDs, especially aspirin (see Ivey, this issue). Symptoms include dyspepsia, heartburn, epigastric pain, nausea and vomiting; objective signs of gastrointestinal irritation reported most often are gastric erythema, pinpoint haemorrhages, mucosal erosions, occult blood loss, exacerbation of gastric ulceration and, more seriously, severe gastric ulceration and frank haemorrhage (for reviews see Ivey, 1983; Piper, 1983).

The relative incidence of gastrointestinal side effects caused by aspirin is low; it has been estimated that between 2 and 6% of patients will develop dyspepsia, nausea and vomiting (Editorial, British Medical Journal, 1981). Additionally, epidemiological surveys have demonstrated an increased risk of severe haemorrhage or gastric ulceration only in persons taking aspirin in large doses (more than 15 tablets per week) or regularly (4 or more days per week). Such adverse consequences are rare: their respective incidences have been calculated as 10 and 15 per 100,000 habitual aspirin users (see Editorial, British Medical Journal, 1981; Levy, 1974; Rees and Turnberg, 1980). For a discussion of the gastrointestinal toxicity of aspirin and salicylates see Ivey, this issue.

The mechanisms by which NSAIDs cause gastrointestinal disturbances are complex and are discussed by Ivey in this issue. At the present time,

inhibition of prostaglandin synthesis seems fundamental to the changes that occur, and such a mechanism would explain why all the acidic NSAIDs share gastrointestinal irritancy as their most frequent adverse effect.

In various animal species, including man, prostaglandins (particularly of the E series) have been demonstrated to inhibit gastric acid secretion, increase mucosal blood flow, and have a cytoprotective role (Hanks, 1982; Vane, 1975). It follows that peripheral cyclo-oxygenase inhibitors, which reduce the biosynthesis of prostaglandins (see section 1.1), probably increase the risk of gastric mucosal damage. Other mechanisms which may contribute to the adverse effects of aspirin in the stomach include a direct irritant action and its ability to interfere with platelet function (see section 4.4). The relative importance of the local and systemic effects of aspirin in the pathogenesis of gastrointestinal injury remains debatable, and further research is needed to clarify the exact mechanisms involved.

Numerous formulations of aspirin and many new derivatives of salicylic acid have been evaluated to try and reduce gastrointestinal complaints while maintaining analgesic efficacy. From studies published to date, a number of conclusions can be reached:

1. Plain unbuffered formulations of aspirin cause the greatest amount of gastrointestinal damage.
2. Other formulations of aspirin, such as soluble, adequately buffered, enteric-coated and extended-release, reduce the incidence of gastrointestinal effects but not to an extent that they can be recommended without reservation for use in patients with upper gastrointestinal problems such as dyspepsia.
3. Newer non-narcotic analgesics related to salicylic acid, like choline magnesium trisalicylate, salsalate, fendosal, fosfosal and diflunisal, also appear to have significantly less effect on the gastrointestinal tract. However, although such drugs produce a lower overall incidence of adverse gastric reactions, gastrointestinal toxicity seems inevitable owing to their actions on cyclo-oxygenase,

and they should be used with extreme caution in patients with suspected gastrointestinal disease.

4.2 Renal Effects

Generally, renal function is not adversely affected by intermittent administration of usual dosages of aspirin and related salicylates. However, there is extensive clinical evidence linking analgesic abuse with chronic renal disease (see Kincaid-Smith, this issue). The characteristic lesion is renal papillary necrosis with secondary cortical damage leading to progressive renal failure (for an extensive review see Prescott, 1982). Analgesic nephropathy is, in the majority of instances, associated with chronic analgesic abuse, especially with combinations of various non-prescription drugs.

From the outset, phenacetin was considered the main instigator of analgesic-induced changes in kidney function, although the evidence implicating as the sole nephrotoxic agent appears circumstantial. Indeed, removal of phenacetin from several of the world's markets has not been followed by the expected reductions in mortality from renal disease. As emphasised by Prescott (1982), serious doubt must be cast on the supposed role of phenacetin as the major aetiological agent associated with analgesic nephropathy. The part played by non-narcotic analgesics such as aspirin and other acidic anti-inflammatory drugs should also be considered fundamental to the development of renal injury. Animal studies have confirmed that chronic administration of aspirin produces renal damage and, in man, salicylates can produce a transient increase in the urinary excretion of renal tubular epithelial cells, increases in blood urea nitrogen, and proteinuria. Furthermore, more than 150 examples of analgesic nephropathy have been reported in patients taking aspirin without phenacetin. Putting the renal effects of aspirin and related salicylates into some perspective, their overall actions seem relatively minor; usual analgesic doses, even when administered for years, have rarely produced any serious effects on the kidney. However, chronic use of anti-inflammatory doses, or abuse, have produced a high incidence of renal papillary necrosis

Table II. Summary of reported incidence of salicylate-induced hepatic injury calculated by Zimmerman (1981) in healthy subjects and patients with various rheumatic and collagen diseases

Clinical diagnosis	Incidence of salicylate-induced hepatic injury (%)
Normal subjects and patients with non-rheumatic disease	0 to 30
Adult rheumatoid arthritis	20
Systemic lupus erythematosus ^a	47
Rheumatic fever	50 to 70
Juvenile rheumatoid arthritis ^a	25 to 70

a Active disease has higher incidence and greater severity of injury than 'inactive' disease.

in patients with rheumatoid arthritis and in analgesic abusers. The precise mechanisms causing renal damage are not known, although inhibition of renal prostaglandin synthesis has been postulated and would explain why the acidic anti-inflammatory analgesics share the potential for nephrotoxicity. Since aspirin inhibits cyclo-oxygenase, it should be used with caution in patients with impaired renal function and those likely to have increased dependence upon renal prostaglandins for maintenance of renal blood flow (e.g. those with congestive heart failure, ascites, systemic lupus erythematosus).

4.3 Hepatic Effects

It was not until fairly recently that the hepatotoxic effects of aspirin were first suspected – after more than 50 years of clinical use. Generally, aspirin-induced liver injury develops after 1 to 4 weeks' treatment with relatively large doses of aspirin, and in most cases it is mild and reversible. Hepatotoxicity appears to be related to serum salicylate concentrations, usually occurring at levels in excess of 200 to 250 mg/L (Zimmerman, 1981; Prescott, this issue).

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kaline phosphatase are occasionally found. Plasma bilirubin concentrations may be elevated – with jaundice in approximately 3% of patients. Blood prothrombin concentrations have infrequently been decreased to an extent that caused an increase in prothrombin time. Many patients are asymptomatic, although some develop nausea, vomiting, anorexia, abdominal pain, liver tenderness and/or hepatomegaly.

Although aspirin-induced hepatotoxicity has been reported in healthy subjects, it most frequently develops in patients with inflammatory diseases (table II). These patients usually receive high doses of aspirin and are more likely to have high salicylate plasma concentrations.

Diflunisal has been reported to cause occasional reversible elevations in liver function tests and very rarely cholestasis and/or jaundice.

4.4 Haematological Effects

Single oral doses of aspirin 300mg and greater have been demonstrated to inhibit platelet aggregation and prolong bleeding time in healthy individuals (Farah and Rosenberg, 1980; Flower et al., 1980; Mielke, 1981). These effects appear to be mediated through inhibition of cyclo-oxygenase and hence thromboxane A₂ production (Hanks, 1982). Aspirin irreversibly acetylates cyclo-oxygenase, but other salicylates (including diflunisal) act as competitive inhibitors and do not seem to produce such a marked and prolonged inhibition of platelet aggregation (Nitelius et al., 1984).

The above actions of aspirin on platelet function do not normally result in any morbidity in healthy subjects. However, they may be a problem in patients at risk of bleeding, such as those with haemophilia, vitamin K deficiency, hypoprothrombinaemia or hepatic damage and those taking anticoagulants or about to undergo surgery. In addition to these effects, salicylates may cause iron deficiency anaemia as a result of chronic gastrointestinal blood loss.

Serious blood dyscrasias induced by salicylates are extremely rare. Isolated reports have implicated aspirin as a cause of aplastic anaemia and

thrombocytopenia. The first case of aspirin-induced haemolytic anaemia with thrombocytopenia was reported in 1984 (Hubert et al., 1984). Salicylates do not ordinarily alter leucocyte counts.

4.5 Hypersensitivity

Aspirin is one of the most common causes of 'allergic' drug reactions such as asthma, rhinitis, urticaria and angioedema. Systemic anaphylaxis occurs occasionally in patients with pseudo-allergic reactions to aspirin (see Szczeklik, this issue). Estimates of the incidence of aspirin hypersensitivity have been extremely varied, depending on the type of analysis and the type of patient. It is clear that the incidence in historical surveys is much lower than after provocative testing of patients at risk. Furthermore, certain patient groups appear to be much more susceptible (Settipane, 1983) [table III]. Recent studies have revealed that aspirin sensitivity may have different clinical manifestations with different underlying pathogeneses. Two major subtypes of patient have been identified: those who develop a respiratory reaction such as rhinitis and/or asthma, and those who react with the development of urticarial weals and angioneurotic oedema (Hanks, 1982; Settipane, 1983; Szczeklik, 1983).

The allergic response to aspirin usually occurs within minutes of ingestion and almost always

Table III. Approximate frequency of aspirin hypersensitivity in various groups of patients (adapted from Settipane, 1983)

Study group	Frequency of aspirin hypersensitivity (%) ^a
General population	~ 0.3
Rhinitis	~ 1.4
Asthma	4-19
Nasal polyps	14-23
Chronic urticaria	23-28

^a Low frequencies are associated with studies based on historical data; higher frequencies are associated with aspirin challenge studies.

within an hour, although occasionally a delayed reaction may take place. The term allergic implies an immunological mechanism for aspirin hypersensitivity and drug-dependent platelet antibodies have been reported in patients with aspirin-induced haemolytic anaemia or thrombocytopenia (Conti et al., 1984; Hubert et al. 1984). At present the pathogenesis of aspirin intolerance is poorly understood. The ubiquitous *inhibition of cyclooxygenase* has been speculated to result in an increased production of leukotrienes and slow reacting substance of anaphylaxis (SRS-A; fig. 2) but further research is clearly necessary to fully understand the precise mechanisms involved in this idiosyncratic reaction.

Hypersensitivity occurs more frequently with aspirin than with other salicylates. However, patients sensitive to aspirin may also develop cross-sensitivity to non-narcotic analgesics, NSAIDs and azo dyes such as tartrazine. Drugs which may precipitate life-threatening bronchoconstriction, and which are absolutely contraindicated in patients who develop aspirin-induced asthma, include indomethacin, ibuprofen, mefenamic acid, flufenamic and meclofenamic acids, fenoprofen, ketoprofen, naproxen, diclofenac and phenylbutazone. In patients known to be hypersensitive to aspirin it seems clinically prudent to avoid all inhibitors of prostaglandin synthesis (especially NSAIDs) if at all possible, and vice versa. There is some evidence that drugs such as paracetamol, dextropropoxyphene, salicylamide, and various salts of salicylic acid (sodium salicylate, salsalate) do not produce such extensive cross-sensitivity and may be used (with caution) in patients with aspirin-induced asthma.

4.6 Auditory and Vestibular Effects

Anti-inflammatory doses of aspirin (> 2.4 g/day) have been reported to cause tinnitus, deafness, headache or dizziness (Miller, 1982). Symptoms rapidly disappear once the dosage is reduced. Tinnitus and hearing loss are dose related and are considered to be the first signs of chronic salicylate intoxication (salicylism); most patients notice their

onset at salicylate concentrations above about 250 mg/L (see section 4.9).

4.7 Reye's Syndrome

Reye's syndrome is a life-threatening childhood disease which results in acute encephalopathy and fatty degeneration of the liver, and typically follows an acute viral infection; influenza A, influenza B or varicella (Reye et al., 1963). Although 20 to 40% of cases are fatal and there is no known cure for the disease, earlier diagnosis, greater understanding of the disease by medical staff, and intensive supportive care may help to improve the prognosis (Orlowski, 1984).

Current evidence suggests an association between the use of salicylates (particularly aspirin) and the development of Reye's syndrome, but a causal relationship has not been established (see Prescott, this issue).

4.8 Pregnancy and Lactation

Salicylates have been found to be teratogenic in animals, although no evidence for such effects has been reported in prospective mothers in a number of large scale epidemiological surveys (Crombie et al., 1970; Slone et al., 1976; Turner and Collins, 1975). Turner and Collins did, however, find a significantly increased incidence of anaemia, antepartum and postpartum haemorrhage, prolonged gestation and complicated deliveries in mothers who had taken salicylates. Furthermore, in the salicylate group there was an increased stillbirth rate and a significantly reduced birthweight (for reviews see Hanks, 1982; Thomas, 1983a). An increase in peripartur haemorrhage can occur through the effect of salicylates on platelet function (see Heymann, this issue).

Since prostaglandins are known to be involved in the initiation and regulation of labour, it seems likely that salicylates prolong gestation by inhibiting prostaglandin biosynthesis. As with any drug, the benefit to risk ratio should be carefully analysed before administering aspirin to pregnant women. If deemed necessary, low doses may be

given during trimesis, prolonged during Salbreast, tion to doses Thnursing salicyl, been milk.

4.9

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given for analgesic/anti-inflammatory purposes during the first 6 months of pregnancy. In the last trimester aspirin must be avoided because it may prolong labour and/or lead to greater blood loss during delivery.

Salicylates are excreted to a minor extent in breast milk and should be administered with caution to nursing mothers. However, occasional single doses seem to present little risk.

The use of diflunisal in pregnant women and nursing mothers reflects the caution necessary for salicylates in general. Safety in pregnancy has not been established. Diflunisal is excreted in breast milk at about 2 to 7% of the plasma concentration.

4.9 Other Adverse Effects - Toxicity

Usual doses of salicylates have minimal effects on the cardiovascular, central nervous or respiratory systems. However, acute overdosage can lead to gastrointestinal disturbances, tinnitus, deafness, hyperventilation and disturbed acid/base balance - respiratory alkalosis and eventually metabolic acidosis due to uncoupling of oxidative phosphorylation and accumulation of organic acids. More severe toxic effects such as severe hyperventilation, convulsions, cyanosis, coma, oliguria, uraemia, pulmonary oedema and respiratory or cardiovascular failure may occur as the plasma salicylate concentration increases.

Salicylism may also develop with high dose chronic salicylate therapy. Such intoxication is manifested initially as headache, tinnitus, deafness, vertigo, lassitude, sweating, thirst, hyperventilation, nausea, and vomiting. More severe CNS disturbances, marked alterations in acid/base balance, and fever may develop if salicylate is allowed to accumulate in the blood. Children and the elderly are particularly at risk of chronic salicylate intoxication, but rarely from intentional overdosage; it usually occurs accidentally from overuse of a drug which many people still consider to be completely safe. Mortality from chronic salicylate intoxication (about 25%) is considerably higher than that reported after acute overdose (1 to 2%) [Proudfoot, 1983].

5. Drug Interactions

A large number of drug interactions involving salicylates have been documented but relatively few seem to be clinically important. Drugs which are considered to produce the most significant interactions with salicylates include anticoagulants and thrombolytic agents, sulphonylurea hypoglycaemic agents, uricosuric agents, methotrexate, corticosteroids and some diuretics (Hull Hayes, 1981; Thomas, 1983b).

5.1 Anticoagulants and Thrombolytic Agents

Salicylates have the potential to affect the activity of oral anticoagulants by several mechanisms. Large doses (≥ 3 g/day) occasionally result in a reduction of plasma prothrombin concentration (see section 4.3) and may enhance the hypoprothrombinaemic effects of oral anticoagulants. Salicylates can displace coumarin anticoagulants such as warfarin from their plasma protein binding sites, consequently producing a transient rise in free active anticoagulant.

Probably of greater clinical importance is that salicylates cause gastric erosions and increase gastrointestinal blood loss (see section 4.1). Aspirin, in particular, inhibits platelet aggregation and may cause prolongation of bleeding time (see section 4.4). These findings militate against the concomitant administration of salicylates with drugs such as anticoagulants, streptokinase, urokinase and heparin. Obviously, administration of salicylates in patients treated with anticoagulants or thrombolytic agents is contraindicated.

5.2 Oral Hypoglycaemic Agents

Salicylates may potentiate sulphonylurea-induced hypoglycaemia (e.g. with chlorpropamide, tolbutamide) and the combination is best avoided. The precise mechanisms involved are not known.

5.3 Uricosuric Agents

Aspirin and related salicylates antagonise the uricosuric effects of probenecid, sulphinpyrazone

and phenylbutazone. The most likely explanations for these interactions appear to involve changes in plasma protein binding and/or competition for renal routes of elimination. It seems clinically prudent to avoid the concomitant use of salicylates with these drugs.

5.4 Methotrexate

Salicylates increase the toxic effects of methotrexate when the 2 drugs are co-administered and, again, the mechanism appears to be related to direct competition of the drugs in question for plasma binding sites and/or active renal transport sites. Because methotrexate has a low therapeutic index, and consequently a narrow margin of safety, this combination of drugs must be avoided.

5.5 Corticosteroids

There is limited clinical evidence that administration of corticosteroids may decrease serum salicylate concentrations when given concomitantly. However, these drugs are frequently used together without deleterious consequences. Special care may be needed when corticosteroids are discontinued, since a rebound increase in plasma salicylate concentration may occur. An isolated case of salicylate toxicity following hydrocortisone withdrawal has been recorded (Klinenberg and Miller, 1965).

5.6 Diuretics

Aspirin has been shown to slightly reduce the natriuretic effects of spironolactone and to attenuate the diuretic effects of furosemide (furosemide) [American Hospital Formulary, 1985; Webster, 1985]. The principal adverse clinical effect of the attenuated natriuretic response to diuretics by NSAIDs is worsening of cardiac failure. Additionally, diflunisal causes a 25 to 30% increase in the plasma concentration of hydrochlorothiazide and a decrease in its urinary excretion. However, the clinical importance of these interactions is not certain (Brogden et al., 1980).

5.7 Other Interactions

Many of the interactions described above are pharmacokinetic interactions that relate to direct competition of salicylates and concomitantly administered drugs for the same plasma protein binding sites and/or for active transport mechanisms within the proximal tubule of the nephron. Other drugs which may interact with salicylates in this way include penicillins, phenytoin, valproic acid, other NSAIDs and some sulphonamides. Salicylates may also displace bilirubin from its albumin binding sites in neonates, with the risk of causing hyperbilirubinaemia.

Food delays the onset and reduces the extent of salicylate (including diflunisal) absorption; however, most aspirin formulations should be taken after food to minimise gastrointestinal toxicity. Concomitant administration of salicylates and ethanol should be avoided since salicylate-induced gastric disturbances may be accentuated by alcohol (see Ivey, this issue).

Because salicylate excretion is pH dependent (see section 2.3), any drug which affects urinary pH may substantially alter salicylate reabsorption/excretion. Thus, antacids, which increase urinary pH and decrease salicylate reabsorption, will lower plasma salicylate concentrations. Drugs which acidify the urine (e.g. ammonium chloride) have the converse effect.

Acetazolamide should be avoided in patients receiving salicylates, since it not only has the potential to increase urinary pH (thus decreasing salicylate reabsorption) but it also induces metabolic acidosis, which would enhance salicylate penetration into the central nervous system and other tissues.

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Percutaneous Absorption of Salicylic Acid

J. Richard Taylor, MD,
Kenneth M. Halprin, MD

The potential hazards of repeated topical application of salicylic acid under occlusion to large areas of the body was evaluated by measuring the percutaneous absorption and serum salicylate concentrations in four patients with active psoriasis.

Serum salicylate concentrations never exceeded 5 mg/100 ml in any of the patients, and although greater than 60% of the salicylic acid applied was absorbed, no evidence of accumulation or toxicity was observed. This form of treatment appears to present little potential hazard even in patients with extensive skin disease. Therapy could be hazardous for patients with impaired hepatic or renal function or for smaller children.

The urinary excretory products of salicylate metabolism were compared following topical and intravenous salicylate administration to determine if the skin plays any part in the biotransformation of salicylate during percutaneous absorption. Our data are too limited and inconclusive to answer this question.

Topical preparations containing salicylic acid have been used in dermatology for many years, and salicylate toxicity resulting from their use has been reported.¹⁻³ Unfortunately there is little information available concerning the potential

hazards of using large quantities of salicylic acid on large areas of the body surface in situations where skin disease exists and occlusive therapy may be desirable. A preparation containing 6% salicylic acid in a gel comprised of 60% propylene glycol and 19.4% alcohol (Keralyt) has recently become available for the treatment of patients with generalized skin disease under occlusion. This study is an attempt to evaluate the potential hazard of using this product. This was accomplished by determining the percutaneous absorption and serum salicylate levels produced when this preparation is used in a well-controlled clinical setting designed to provide maximal exposure. To determine if the degradation of salicylic acid is altered when salicylate is absorbed percutaneously, the products of salicylate metabolism excreted in the urine were compared after topical and intravenous administration.

PATIENTS AND METHODS

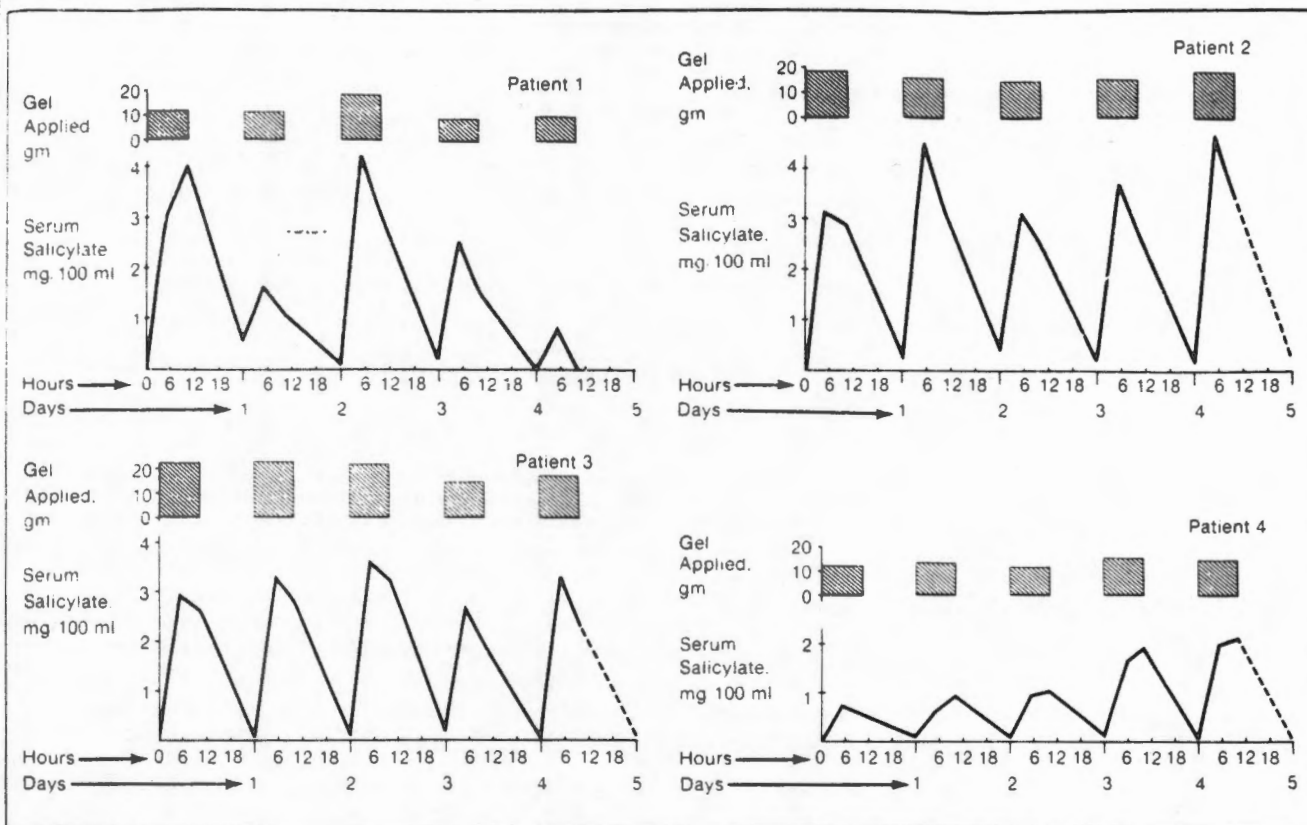
Patients

Four patients with active psoriasis were studied as inpatients at the Miami Veterans Administration Hospital. None of the patients had renal disease, had ingested salicylate-containing medications for one week prior to the study, or was permitted to use other topical or systemic medications for the duration of the study. All of the patients had more than 25% of their body surface involved with psoriasis (patient 2 had severe generalized psoriasis, with more than 90% involvement). In-

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Serum salicylate concentrations and the number of grams of 6% salicylic acid gel applied for days 1 through 5 for each of the four patients are shown. Dotted line connects point representing last salicylate level which was measured (tenth hour of day 5) to base line for illustrative purposes.

formed consent was obtained from each patient after the procedures and hazards of the study had been fully explained.

Patients applied the contents of a 28-gm tube of medication to their entire body surface below the neck immediately after taking a shower. The treated areas were covered with plastic wrap and kept occluded for ten hours. After ten hours, the dressings were removed and the patient allowed to shower. This treatment was repeated daily for a total of five days. All tubes of medication were preweighed and then reweighed after use to determine the grams of gel applied by each patient each day.

One week after completing the above study patient #3 was given 1 liter of physiological saline solution containing 1.0 gm of sodium salicylate intravenously during an eight-hour period.

Collection of Samples

Venous blood was obtained prior to application of the topical medication and five and ten hours after application each day for five days. The serum was separated and stored refrigerated until analyzed.

All of each patient's urine was collected for each 24-hour period starting from the

beginning of the study for a total of seven days. At the end of each day, the urine volume excreted was measured; an aliquot was sent to the hospital clinical laboratory for creatinine determination, and an aliquot was refrigerated for analysis of salicylates.

Serum was obtained in a similar manner from the patient receiving intravenous sodium salicylate, but it was collected at 0, 4, 8, and 24 hours after injection. Urine was collected prior to and for three days following the intravenous administration.

Salicylate Determinations

The serum salicylate level was determined fluorometrically by the method described by Saltzman.⁴

Salicylic acid and its metabolites in urine were determined by the method of Levy and Procknal.⁷ This procedure determines the total grams of salicylate excreted and the percent of this total present as free salicylic acid, salicyluric acid, and the acyl and phenolic glucuronides of salicylate.

RESULTS

The Figure illustrates the serum salicylate concentrations and grams of

gel applied for each of the four patients for the five days that serum samples were collected. In no instance did the serum salicylate concentration exceed 5 mg/100 ml, and the average peak serum concentration for all of the patients was less than 3 mg/100 ml (2.7). The patient with the most severe psoriasis (patient 2) developed the highest serum salicylate concentrations (Figure). The serum salicylate levels had returned to low or undetectable values by 24 hours after administration of the drug, and in no instance did there appear to be an accumulation of salicylate during the five days of treatment. A gradual accumulation of salicylate might be suspected in patient 4 (Figure) because of the progressive increase in his serum salicylate levels, but this was no doubt the result of applying greater quantities of topical medication since his serum levels returned to normal prior to each subsequent day's treatment.

The results of the urine salicylate

analyses are summarized in Table 1. We were able to recover more than 60% of the salicylic acid applied topically in the urine of all four patients. Patient 2 had the greatest percutaneous absorption: but as indicated previously, he also had the most widespread psoriasis. These results indicate that salicylic acid is efficiently released from the vehicle we used, and it encounters very little resistance to penetration through diseased skin under occlusion.

Table 1 also shows the percent of the total salicylate excreted, which is represented by each of the three major metabolites. In table 2, the percent of the total salicylate excreted as each metabolite after topical application of salicylic acid gel is compared to the metabolites found following intravenous sodium salicylate administration for patient 3. These percentages were calculated from the total quantities of each metabolite excreted during the period of the study and not by averaging the percentages of each excreted per day. We have also included in Table 2 the expected percent of each of the salicylate metabolites after oral administration, as reported in the literature,⁸ for purposes of comparison. All of our patients excreted less salicylate as salicyluric acid and more as salicylate glucuronides than expected. Patient 3 exhibited these changes not only following topical application of salicylic acid but also when given sodium salicylate intravenously.

COMMENT

The findings of our study indicate that salicylic acid is rapidly absorbed when applied in a propylene glycol-alcohol gel vehicle under occlusion to diseased skin. When applied under occlusion for ten hours, more than 60% of the salicylic acid applied was absorbed. In three of the four patients, peak serum levels occurred within five hours, despite the fact that occlusion was carried out for ten hours. The serum salicylate concentration never exceeded 5 mg/100 ml, however, which is well below levels of 30 to 40 mg/100 ml associated with systemic toxic reactions.⁹ Since 28 gm of this preparation will easily cover the

Table 1.—Results of Urinary Salicylate Analyses*

Day	Gel Applied, gm	SA Applied, mg	Salicylate Excreted, mg	Urinary Metabolites			
				% SA	% SU	% SG	
Patient 1							
1	12.4	744	634	4	58	38	
2	11.6	696	369	3	53	44	
3	18.3	1098	584	3	57	40	
4	9.4	564	400	2	62	36	
5	10.1	606	309	3	65	32	
6	0	0	69	3	69	28	
7	0	0	5	18	47	35	
Total	61.8	3708	2370				
Absorption: 64%							
Patient 2							
1	18.5	1110	690	14	52	34	
2	16.0	960	848	11	53	36	
3	14.7	882	750	13	54	33	
4	15.3	918	690	4	55	41	
5	18.8	1128	874	4	48	48	
6	0	0	7	—	—	—	
7	0	0	213	1	62	37	
Total	83.3	4998	4072				
Absorption: 82%							
Patient 3							
1	22.3	1338	615	8	58	34	
2	22.6	1356	684	6	56	38	
3	21.8	1308	764	7	52	41	
4	14.4	864	1123	2	41	57	
5	17.2	1032	553	6	54	40	
6	0	0	1	—	—	—	
7	0	0	0	—	—	—	
Total	98.3	5898	3740				
Absorption: 63%							
Patient 4							
1	12.1	726	210	5	57	38	
2	13.5	810	349	0	60	40	
3	12.1	726	326	3	57	40	
4	15.9	954	605	6	56	38	
5	14.8	888	927	4	51	45	
6	0	0	410	3	53	44	
7	0	0	0	—	—	—	
Total	68.4	4104	2827				
Absorption: 69%							

* SA indicates salicylic acid; SU, salicyluric acid; SG, acyl and phenolic glucuronides of salicylic acid; — indicates concentration insufficient for reliable determination.

Table 2.—Salicylate Metabolites Excreted After Different Routes of Administration

Route of Administration	% of Total Urinary Salicylate		
	Free Salicylic Acid	Salicyluric Acid	Salicylate Glucuronides
Topically applied gel containing salicylic acid	6	52	42
Intravenously administered sodium salicylate	3	63	34
Orally administered salicylate*	10	70	20

* Data obtained from Goodman and Gilman.⁸

entire body surface of a large man and occlusive therapy would rarely be used for more than ten hours each day, this form of treatment appears to present little potential hazard even in patients with extensive skin disease.

Absorbed salicylic acid distributes into the extracellular fluid volume (150 ml/kg),⁸ which is about 10 liters in a 72-kg man. A 28-gm tube of 6% salicylic acid contains 1.8 gm of salicylic acid; if 60% of it is absorbed, the maximal serum level that could result would be about 1 gm/10 liters or 10 mg/100 ml, which is well below toxic concentrations. Toxic reactions would not have occurred in this study even if 100% of the applied salicylic acid had been absorbed since the expected serum level (18 mg/100 ml) would still be below a toxic level. We did not believe it was necessary to produce a toxic reaction to salicylate to adequately determine the percutaneous penetration of salicylic acid, and the study was designed to minimize this possibility.

Topical salicylic acid therapy can be hazardous, however, and salicylate toxicity as well as death from acute salicylate poisoning can occur from its injudicious use. To prevent a toxic reaction, three factors should be kept in mind. The quantity of salicylic acid used should not be excessive, the available extracellular fluid volume should not be limited, and the ability to metabolize and excrete the absorbed medication should not be im-

paired. Excessive quantities of salicylic acid can result from either a high concentration in the medication or from frequently repeated applications to large areas of the body. The two fatal cases reported by Lindsey⁴ occurred after only two applications of an antifungal solution containing 20.7% salicylic acid to 50% of the body surface area. vonWeiss and Lever³ reported a toxic reaction to salicylate in three patients treated with 3% and 6% salicylic acid ointment applied six times daily for 4 to 11 days.

Toxic reactions to salicylate occur more frequently in smaller children because their extracellular fluid volume is much smaller in comparison to the potential surface area available for treatment. A contracted extracellular volume is also a potential hazard in dehydrated debilitated patients. Such patients can also develop a toxic reaction to salicylate as a result of impaired excretion if hepatic or renal disease exists. These factors may have contributed to the toxic reaction to salicylate reported in some cases,^{2,4} although they were not specifically discussed. The cases reviewed by Young,¹ however, demonstrate the increased risk of treating children.

Most of the salicylic acid absorbed is metabolized to more water soluble products to facilitate its excretion. Salicylic acid is conjugated with glycine, forming salicyluric acid, which normally accounts for 70% of the salicylate excreted. It is also conjugated with glucuronic acid forming several

salicylate glucuronides, which as a group account for 20%; the remainder is excreted as free salicylic acid. Although the metabolism of salicylate occurs primarily in the liver, the intestinal mucosa can also synthesize salicylate glucuronides.¹⁰ We wondered if the skin might also possess the glucuronyl transferase system necessary for this conjugation. This would explain the higher than expected urinary salicylate glucuronide levels found in our patients. To further study this possibility, we administered salicylate intravenously in one of our patients to see what percent of the excreted salicylate was salicylate glucuronide when its absorption did not occur through either the intestinal mucosa or skin.

We expected that intravenous administration, which would by-pass possible conjugation systems in both the gut and the skin, would result in a lower level of glucuronides than after topical (or oral) administration. Table 2 shows that the amount of salicylate excreted as glucuronides was lower than after topical application, but it was still fairly high and leaves open the question of the possible glucuronide conjugation of drugs by the skin. This question should be approached by more direct biochemical methods for the demonstration of the presence and in vivo activity of this drug metabolizing system.

The salicylic acid used in this investigation was supplied as Keralyt by Westwood Pharmaceuticals Inc., Buffalo, NY.

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When dealing with a child, the choice of drugs is considerably restricted, and only drugs that have been extensively tested in children should be used. This commonly means that only aspirin, naproxen, or tolmetin should be prescribed. However, the association of Reye's syndrome in children with the administration of aspirin for the treatment of febrile viral illnesses precludes its use in this setting. The use of any of the aspirin-like drugs in pregnant women is generally not recommended. If such a drug must be given to a pregnant woman, low doses of aspirin are probably the safest. Although toxic doses of salicylates cause teratogenic effects in animals, there is no evidence to suggest that salicylates in moderate doses have teratogenic effects on the human fetus. In any case, aspirin should be discontinued prior to the anticipated time of parturition in order to avoid complications such as prolongation of labor, increased risk of postpartum hemorrhage, and intrauterine closure of the ductus arteriosus.

Many aspirin-like drugs bind firmly to plasma proteins and thus may displace certain other drugs from the binding sites. Such interactions can occur in patients given salicylates or phenylbutazone together with warfarin, a sulfonyleurea hypoglycemic agent, or methotrexate; the dosage of such agents may require adjustment, or concurrent administration should be avoided. The problem with warfarin is accentuated because almost all of the aspirin-like drugs disturb normal platelet function.

Initially, fairly low doses of the agent chosen should be prescribed to determine the patient's reaction. When the patient has problems with sleeping because of pain or morning stiffness, a larger single dose of the drug may be given at night; as an alternative, single doses of another drug (e.g., 50 to 100 mg of indomethacin) may be given to supplement existing medication without much danger of serious side effects. A week is generally long enough to determine the effect of a given drug. If the drug is effective, treatment should be continued, reducing the dose if possible and stopping it altogether if it is no longer necessary. Side effects usually appear in the first weeks of therapy. If the patient does not respond, another compound should be tried, since there is a marked variation in the response of individuals to different but closely related drugs.

For mild arthropathies, the scheme outlined above, together with rest and physical therapy, will probably be effective. However, patients with a

more debilitating disease may not respond adequately. In such cases, more aggressive therapy should be initiated with aspirin or another agent. It is best to avoid continuous combination therapy with more than one aspirin-like drug; there is no evidence of extra benefit to the patient, and the incidence of side effects is generally additive.

For the seriously debilitated patient who cannot tolerate these drugs or in whom they are not adequately effective, other forms of therapy should be considered. Gold, hydroxychloroquine, and penicillamine are discussed in a separate section of this chapter. Other relevant drugs include immunosuppressive agents (Chapter 53) and glucocorticoids (Chapter 60).

A final important consideration is the cost of therapy, particularly since these agents are frequently used on a long-term basis. Generally speaking, aspirin is very inexpensive, ibuprofen has become less costly than phenylbutazone, and indomethacin, and the cost of the newer drugs can be very high.

THE SALICYLATES

Despite the introduction of many new drugs, aspirin (acetylsalicylic acid) is still the most widely prescribed analgesic-antipyretic and antiinflammatory agent, and it is the standard for the comparison and evaluation of the others. Prodigious amounts of the drug are consumed in the United States; some estimates place the quantity as high as 10 to 20 thousand tons annually. The layman relies upon it as the common household analgesic; yet, because the drug is so generally available, its usefulness is often underrated. Despite the efficacy and safety of aspirin as an analgesic and antirheumatic agent, it is necessary to be aware of its role in Reye's syndrome and as a common cause of lethal drug poisoning in young children, as well as its potential for serious toxicity if used improperly.

The older literature on salicylates has been summarized by Hanzlik (1927). More recent reviews of some of the clinical pharmacology appear in several symposia (1983a, 1983c) and in a monograph (Rainsford, 1985a).

Chemistry. Salicylic acid (orthohydroxybenzoic acid) is so irritating that it can only be used externally; therefore, various derivatives of this acid have been synthesized for systemic use. These comprise two large classes, namely, esters of salicylic acid obtained by substitution in the carboxyl group and salicylate esters of organic acids in which the carboxyl group of salicylic acid is re-

placed and substitution is made. For example, aspirin is an ester. In addition, there are salts of salicylic acid. Relationships can be seen in the formulas shown in Table 26-1.

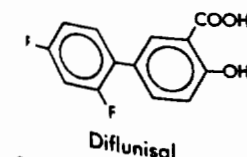
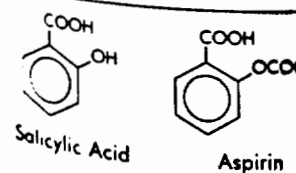
Structure-Activity Relationship. Salicylates generally act by virtue of their acidic nature, and although some of the unionized form are due to its capacity to accept protons. Substitutions on the carboxyl group change the potency or duration of action. The *ortho* position of the carboxyl group is an important feature for the action of salicylic acid, $C_6H_4(COOH)_2$, shares the same activity as salicylic acid but is much weaker. Simple substitutions on the benzene ring are extensively studied, and new ones are still being synthesized. A nonsteroidal anti-inflammatory, diflunisal, is also available.

PHARMACOLOGICAL PROPERTIES

Analgesia. As noted above, pain is usually relieved by those of low intensity than those of high intensity. The chemical structures rather than the chemical names are important, especially headache, neuralgia, and rheumatism. The salicylates are used for pain relief than is any other class of drugs. Long-term use does not cause tolerance or addiction, and toxicity is less than that of opioid analgesics. Salicylates alleviate pain by virtue of their analgesic action (see above); direct action on the CNS may also be involved.

Antipyresis. As discussed above, salicylates usually lower elevated temperatures rapidly and effectively.

Table 26-1. STRUCTURE-Activity Relationships OF THE SALICYLATES



tained and substitution is made in the OH group. For example, aspirin is an ester of acetic acid. In addition, there are salts of salicylic acid. The chemical relationships can be seen from the structural formulas shown in Table 26-1.

Structure-Activity Relationship. Salicylates generally act by virtue of their content of salicylic acid, although some of the unique effects of aspirin are due to its capacity to acetylate proteins (see below). Substitutions on the carboxyl or hydroxyl groups change the potency or toxicity of the compound. The *ortho* position of the OH group is an important feature for the action of salicylate. Benzoic acid, C_6H_5COOH , shares many of the actions of salicylic acid but is much weaker. The effects of simple substitutions on the benzene ring have been extensively studied, and new salicylate derivatives are still being synthesized. A difluorophenyl derivative, diflunisal, is also available for clinical use.

PHARMACOLOGICAL PROPERTIES

Analgesia. As noted above, the types of pain usually relieved by salicylates are those of low intensity that arise from integumental structures rather than from viscera, especially headache, myalgia, and arthralgia. The salicylates are more widely used for pain relief than is any other class of drugs. Long-term use does not lead to tolerance or addiction, and toxicity is lower than that of opioid analgesics. The salicylates alleviate pain by virtue of a peripheral action (see above); direct effects on the CNS may also be involved.

Antipyresis. As discussed above, salicylates usually lower elevated body temperatures rapidly and effectively. How-

ever, moderate doses that produce this effect also increase oxygen consumption and metabolic rate. In toxic doses, these compounds have a pyretic effect that results in sweating; this enhances the dehydration that occurs in salicylate intoxication (see below).

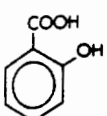
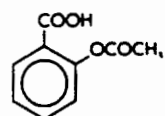
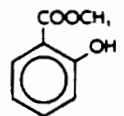
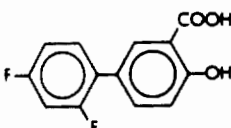
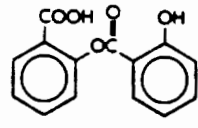
Miscellaneous Neurological Effects. In high doses, salicylates have toxic effects on the CNS, consisting of stimulation (including convulsions) followed by depression. Confusion, dizziness, tinnitus, high-tone deafness, delirium, psychosis, stupor, and coma may occur. The tinnitus and hearing loss caused by salicylate poisoning are due to increased labyrinthine pressure or an effect on the hair cells of the cochlea. Tinnitus is typically observed at salicylate concentrations of 200 to 450 $\mu g/ml$, and there is a close relation between the extent of hearing loss and the concentration of salicylate in plasma. The symptoms are completely reversible within 2 or 3 days after withdrawal of the drug.

Salicylates induce nausea and vomiting, which result from stimulation of sites that are accessible from the cerebrospinal fluid (CSF), probably in the medullary chemoreceptor trigger zone (CTZ). In man, centrally induced nausea and vomiting generally appear at plasma salicylate concentrations of about 270 $\mu g/ml$, but these same effects may occur at much lower plasma values as a result of local gastric irritation.

Respiration. The effects of salicylate on respiration are important because they contribute to the serious acid-base balance disturbances that characterize poisoning by this class of compounds. Salicylates stimulate respiration directly and indirectly. Full therapeutic doses of salicylates increase oxygen consumption and CO_2 production (especially in skeletal muscle); these effects are a result of salicylate-induced uncoupling of oxidative phosphorylation (see below). The increased production of CO_2 stimulates respiration. The increased alveolar ventilation balances the increased CO_2 production, and thus plasma CO_2 tension (P_{CO_2}) does not change. The initial increase in alveolar ventilation is characterized mainly by an increase in depth of respiration and only a slight increase in rate. If the respiratory response to CO_2 has been depressed by the administration of a barbiturate or an opioid, salicylates will cause a marked increase in plasma P_{CO_2} and respiratory acidosis.

Salicylate directly stimulates the respira-

Table 26-1. STRUCTURAL FORMULAS OF THE SALICYLATES

		
Salicylic Acid	Aspirin	Methyl Salicylate
		
Diflunisal	Salsalate	

tory center in the medulla. This results in marked hyperventilation, characterized by an increase in depth and a pronounced increase in rate. Patients with salicylate poisoning may have prominent increases in respiratory minute volume, and respiratory alkalosis ensues. Plasma salicylate concentrations of 350 $\mu\text{g/ml}$ are nearly always associated with hyperventilation in man, and marked hyperpnea occurs when the level approaches 500 $\mu\text{g/ml}$.

A depressant effect of salicylate on the medulla appears after high doses or after prolonged exposure. Toxic doses of salicylates cause central respiratory paralysis as well as circulatory collapse secondary to vasomotor depression. Since enhanced CO_2 production continues, respiratory acidosis ensues (see below).

Acid-Base Balance and Electrolyte Pattern. Therapeutic doses of salicylate produce definite changes in the acid-base balance and electrolyte pattern. The initial event, as discussed above, is respiratory alkalosis. Compensation for the respiratory alkalosis is achieved by increased renal excretion of bicarbonate, which is accompanied by Na^+ and K^+ ; plasma bicarbonate is thus lowered, and blood pH returns toward normal. This is the stage of compensated respiratory alkalosis. This stage is most often seen in adults given intensive salicylate therapy and seldom proceeds further.

Subsequent changes in acid-base status generally occur only when toxic doses of salicylates are ingested by infants and children and occasionally after large doses in adults. In infants and children, the phase of respiratory alkalosis may not be observed, since the child with salicylate intoxication is rarely seen early enough. The stage generally present is characterized by a decrease in blood pH, a low plasma bicarbonate concentration, and a normal or nearly normal plasma P_{CO_2} ; except for the P_{CO_2} , these changes resemble those of metabolic acidosis. However, in reality there is a combination of respiratory acidosis and metabolic acidosis produced as follows. The enhanced production of CO_2 outstrips its alveolar excretion because of direct salicylate-induced depression of respiration;

consequently, plasma P_{CO_2} increases and blood pH decreases. Since the concentration of bicarbonate in plasma is already low because of increased renal bicarbonate excretion, the acid-base status at this stage is essentially an uncompensated respiratory acidosis. Superimposed, however, is a true metabolic acidosis caused by accumulation of acids as a result of three processes. First, toxic concentrations of salicylates displace about 2 to 3 mEq per liter of plasma bicarbonate. Second, vasomotor depression caused by toxic doses of salicylate impairs renal function with consequent accumulation of strong acids of metabolic origin, namely, sulfuric and phosphoric acids. Third, organic acids accumulate secondary to salicylate-induced derangement of carbohydrate metabolism, especially pyruvic, lactic, and acetoacetic acids.

The series of events that produce acid-base disturbances in salicylate intoxication also cause alterations of water and electrolyte balance. The low plasma P_{CO_2} leads to decreased renal tubular reabsorption of bicarbonate and increased renal excretion of Na^+ , K^+ , and water (see introduction to Section VI). In addition, water is lost by salicylate-induced sweating and by insensible water loss through the lungs during hyperventilation, and dehydration rapidly occurs. Since more water than electrolyte is lost through the lungs and by sweating, the dehydration is associated with hyponatremia. Prolonged exposure to high doses of salicylate also causes depletion of K^+ due to both renal and extrarenal factors.

Cardiovascular Effects. Ordinary therapeutic doses of salicylates have no important direct cardiovascular actions. The peripheral vessels tend to dilate after large doses because of a direct effect on their smooth muscle. Toxic amounts depress the circulation directly and by central vasomotor paralysis.

In patients given large doses of sodium salicylate or aspirin, such as the doses used in acute rheumatic fever, the circulating plasma volume increases (about 20%), the hematocrit falls, and cardiac output and work are increased. Consequently, in patients with clear evidence of carditis, such alterations can cause congestive failure and pulmonary edema. High doses of salicylates can also produce noncardiogenic pulmonary edema, particularly in older patients who are ingesting salicylates regularly over a long term.

Gastrointestinal Effects. The ingestion of salicylate may result in epigastric distress, nausea, and vomiting. The mechanism of the emetic effect is discussed above. Salicylate may also

exacerbate (heartburn, hemorrhage, been reported apy, but may as a hyperser induced gastr may lead to a

The daily inge that produces p the usual range to 350 $\mu\text{g/ml}$, re of about 3 to 8 proximately 0.6 (Leonards and L examination in discrete ulcerativ gastric mucosa; rhagic lesions w focal necrosis a bleeding is high slowly and depos sal folds.

As discussed a salicylates injure (see Ivey, in Syr fects result from sion" of acid), w and the submuc necrosis and blee to inhibition of p creased acid secr duction). There m to bleed because

Hepatic and can produce at injury. In one l dependent and plasma concentr above 150 $\mu\text{g/ml}$ cases occur in r sue disorders. toms, and eleva activities in plas tions of hepatic patients also ha and nausea, an in these instance continued beca of fatal hepatic other reasons, r been advised in disease. Considerable of salicylates a severe hepatic

ylate may also cause gastric ulceration; exacerbation of peptic ulcer symptoms (heartburn, dyspepsia), gastrointestinal hemorrhage, and erosive gastritis have all been reported in patients on high-dose therapy, but may occur rarely with low doses as a hypersensitivity response. Salicylate-induced gastric bleeding is painless and may lead to an iron-deficiency anemia.

The daily ingestion of 4 or 5 g of aspirin, a dose that produces plasma salicylate concentrations in the usual range for antiinflammatory therapy (120 to 350 $\mu\text{g/ml}$), results in an average fecal blood loss of about 3 to 8 ml per day as compared with approximately 0.6 ml per day in untreated subjects (Leonards and Levy, 1973). Gastroscopic or direct examination in salicylate-treated subjects reveals discrete ulcerative and hemorrhagic lesions of the gastric mucosa; in many cases, multiple hemorrhagic lesions with sharply demarcated areas of focal necrosis are observed. The incidence of bleeding is highest with salicylates that dissolve slowly and deposit as particles in the gastric mucosal folds.

As discussed above, the mechanisms by which salicylates injure gastric mucosal cells are complex (see Ivey, in Symposium, 1988a). Deleterious effects result from local actions (e.g., "back diffusion" of acid), which cause injury to mucosal cells and the submucosal capillaries with subsequent necrosis and bleeding, and from effects secondary to inhibition of prostaglandin synthesis (e.g., increased acid secretion and decreased mucus production). There may also be an increased tendency to bleed because of impaired platelet aggregation.

Hepatic and Renal Effects. Salicylates can produce at least two forms of hepatic injury. In one form, hepatotoxicity is dose dependent and is usually associated with plasma concentrations that are maintained above 150 $\mu\text{g/ml}$. The vast majority of cases occur in patients with connective tissue disorders. There are usually no symptoms, and elevated enzyme (transaminase) activities in plasma are the principal indications of hepatic damage. About 5% of the patients also have hepatomegaly, anorexia, and nausea, and jaundice may be present; in these instances, salicylates should be discontinued because of the potential hazard of fatal hepatic necrosis. For these and other reasons, restriction of salicylates has been advised in patients with chronic liver disease.

Considerable evidence implicates the use of salicylates as an important factor in the severe hepatic injury and encephalopathy

observed in Reye's syndrome (see Heubi *et al.*, 1987; Hurwitz *et al.*, 1987; Pinsky *et al.*, 1988). This syndrome is a rare but often fatal consequence of infection with varicella and various other viruses, especially the influenza virus. Although a causal relationship between salicylates and Reye's syndrome has not been established, there is a strong epidemiological association. It has been proposed that aspirin and the viral illness may act to damage mitochondria, perhaps preferentially in genetically predisposed individuals (Heubi *et al.*, 1987; Pinsky *et al.*, 1988). The use of salicylates in children or adolescents with chickenpox or influenza is contraindicated.

As discussed above, salicylates can cause retention of salt and water as well as acute reduction of renal function in patients with congestive heart failure or hypovolemia. Although long-term use of salicylates alone is rarely associated with nephrotoxicity, the prolonged and excessive ingestion of analgesic mixtures containing salicylates in combination with acetaminophen or salicylamide can produce papillary necrosis and interstitial nephritis (Clive and Stoff, 1984).

Uricosuric Effects. The effects of salicylates on uric acid excretion are markedly dependent on dose. Low doses (1 or 2 g per day) may decrease urate excretion and elevate plasma urate concentrations; intermediate doses (2 or 3 g per day) usually do not alter urate excretion; large doses (over 5 g per day) induce uricosuria and lower plasma urate levels. Such large doses are poorly tolerated. Even small doses of salicylate can block the effects of probenecid and other uricosuric agents that decrease tubular reabsorption of uric acid (see Chapter 30).

Effects on the Blood. Ingestion of aspirin by normal individuals causes a definite prolongation of the bleeding time. For example, a single dose of 0.65 g of aspirin approximately doubles the mean bleeding time of normal persons for a period of 4 to 7 days. This effect is probably due to acetylation of platelet cyclooxygenase and the consequent reduced formation of TXA_2 .

Patients with severe hepatic damage, hypoprothrombinemia, vitamin K deficiency, or hemophilia should avoid aspirin because the inhibition of platelet hemostasis can result in hemorrhage. If conditions

permit, aspirin therapy should be stopped at least 1 week prior to surgery; care should also be exercised in the use of aspirin during long-term treatment with oral anticoagulant agents because of the possible danger of blood loss from the gastric mucosa. However, the intentional use of aspirin is being investigated for the prophylaxis of thromboembolic disease, especially in the coronary and cerebral circulation (see Reilly and Fitzgerald, 1988; see also Chapter 55).

Salicylates do not ordinarily alter the leukocyte, platelet, or erythrocyte count, the hematocrit, or the hemoglobin content. In acute rheumatic fever, salicylate therapy can reduce leukocytosis and the elevated erythrocyte sedimentation rate. The plasma iron concentration is markedly decreased and erythrocyte survival time is shortened by doses of 3 to 4 g per day. Aspirin is included among the drugs that can cause a mild degree of hemolysis in individuals with a deficiency of glucose-6-phosphate dehydrogenase.

Effects on Rheumatic, Inflammatory, and Immunological Processes, and on Connective Tissue Metabolism. For almost 100 years the salicylates have retained their preeminent position in the treatment of the rheumatic diseases. Although they suppress the clinical signs and even improve the histological picture in acute rheumatic fever, subsequent tissue damage such as cardiac lesions and other visceral involvement is unaffected. In addition to their action on prostaglandin biosynthesis, the mechanism of action of the salicylates in rheumatic disease may also involve effects on other cellular and immunological processes in mesenchymal and connective tissues.

Because of the known relationship between rheumatic fever and immunological processes, attention has been directed to the capacity of salicylates to suppress a variety of antigen-antibody reactions. These include the inhibition of antibody production, of antigen-antibody aggregation, and of antigen-induced release of histamine. Salicylates also induce a nonspecific stabilization of capillary permeability during immunological insults. The concentrations of salicylates needed to produce these effects are high, and the relationship of these effects to the antirheumatic efficacy of salicylates is yet to be determined.

Salicylates can also influence the metabolism of connective tissue, and these effects may be involved in their antiinflammatory action. For example, salicylates can affect the composition, bio-

synthesis, or metabolism of connective tissue mucopolysaccharides in the ground substance that provides barriers to spread of infection and inflammation.

Metabolic Effects. The salicylates have multiple effects on metabolic processes, some of which have already been discussed. Only a few pertinent aspects will be presented here.

Oxidative Phosphorylation. The uncoupling of oxidative phosphorylation by salicylate is similar to that induced by 2,4-dinitrophenol. The effect may occur with doses of salicylate used in the treatment of rheumatoid arthritis and can result in the inhibition of a number of adenosine triphosphate (ATP)-dependent reactions. Other consequences include the salicylate-induced increase in oxygen uptake and carbon dioxide production described above, the depletion of hepatic glycogen, and the pyretic effect of toxic doses of salicylate. Salicylate in toxic doses may decrease aerobic metabolism as a result of inhibition of various dehydrogenases, by competing with the pyridine nucleotide coenzymes, and inhibition of some oxidases that require nucleotides as coenzymes, such as xanthine oxidase.

Carbohydrate Metabolism. Large doses of salicylates may cause hyperglycemia and glycosuria and deplete liver and muscle glycogen; these effects are partly explained by the release of epinephrine. Such doses also reduce aerobic metabolism of glucose, increase glucose-6-phosphatase activity, and promote the secretion of glucocorticoids.

Nitrogen Metabolism. Salicylate in toxic doses causes a significant negative nitrogen balance, characterized by an aminoaciduria. Although adrenocortical activation may contribute to the negative nitrogen balance by enhancing protein catabolism, the mechanism of the aminoaciduria produced by salicylates is poorly understood.

Fat Metabolism. Salicylates reduce lipogenesis by partially blocking incorporation of acetate into fatty acids; they also inhibit epinephrine-stimulated lipolysis in fat cells and displace long-chain fatty acids from binding sites on human plasma proteins. The combination of these effects leads to increased entry and enhanced oxidation of fatty acids in muscle, liver, and other tissues, and to decreased plasma concentrations of free fatty acids, phospholipid, and cholesterol; the oxidation of ketone bodies is also increased.

Endocrine Effects. Adrenal Cortex. Very large doses of salicylate stimulate steroid secretion by the adrenal cortex through an effect on the hypothalamus and transiently increase plasma concentrations of free adrenocorticosteroids by displacement from plasma proteins. However, it is clear that the antiinflammatory effects of salicylate are independent of these effects on adrenocorticosteroids.

Thyroid Gland. Long-term administration of salicylate decreases thyroidal uptake and clearance of iodine, but increases oxygen consumption and rate of disappearance of thyroxine and triiodothyronine from the circulation. These effects are probably due to the competitive displacement by

salicylate of thyroxine and transthyretin and the thyroxine in plasma.

Salicylates and Pregnancy. Salicylates that moderate therapeutic doses cause fetal damage in human babies born to women who in periods may have significant birth. In addition, there is a mortality, anemia, antepartum hemorrhage, prolonged gestation, and deliveries (see above).

Local Irritant Effects. Salicylates irritate skin and mucous membranes. The keratolytic action is employed for the local treatment of fungal infections, and certain dermatitis. The tissue cells desquamate. The salts of salicylates are toxic to the unbroken skin; but they are released in the stomach, and they irritate. Methyl salicylate is irritating to both skin and mucous membranes and is only used externally.

Pharmacokinetics

These important aspects have been reviewed by

Absorption. Orally administered salicylates are absorbed rapidly, particularly in the stomach but mostly from the small intestine. Appreciable concentrations in plasma in less than 1 hour after a single dose, a peak concentration is reached about 2 hours and then declines. Rate of absorption is determined by several factors, particularly the dissolution rates if taken as tablets, and the mucosal surface area and its time.

Salicylate absorption occurs primarily of nondissociated salicylic acid across gastric mucosa and hence is influenced by gastric pH and hence is influenced by salicylate is more ionized at a higher pH also increases the rate of absorption and the overall effect is to increase the rate of absorption. As a result, there is little difference between the rates of absorption of aspirin, and the numerous salicylates. The presence of food delays the absorption of salicylates.

Rectal absorption of salicylates is incomplete, and unreliable; therefore not advisable when the concentrations of the drug are required to be rapidly absorbed from the rectum when applied in oily liniments. Systemic poisoning has occurred.

salicylate of thyroxine and triiodothyronine from transthyretin and the thyroxine-binding globulin in plasma.

Salicylates and Pregnancy. There is no evidence that moderate therapeutic doses of salicylates cause fetal damage in human beings; however, babies born to women who ingest salicylates for long periods may have significantly reduced weights at birth. In addition, there is an increase in perinatal mortality, anemia, antepartum and postpartum hemorrhage, prolonged gestation, and complicated deliveries (see above).

Local Irritant Effects. Salicylic acid is quite irritating to skin and mucosa and destroys epithelial cells. The keratolytic action of the free acid is employed for the local treatment of warts, corns, fungal infections, and certain types of eczematous dermatitis. The tissue cells swell, soften, and desquamate. The salts of salicylic acid are innocuous to the unbroken skin; however, if the free acid is released in the stomach, the gastric mucosa may be irritated. Methyl salicylate (oil of wintergreen) is irritating to both skin and gastric mucosa and is only used externally.

Pharmacokinetics and Metabolism. These important aspects of the salicylates have been reviewed by Davison (1971).

Absorption. Orally ingested salicylates are absorbed rapidly, partly from the stomach but mostly from the upper small intestine. Appreciable concentrations are found in plasma in less than 30 minutes; after a single dose, a peak value is reached in about 2 hours and then gradually declines. Rate of absorption is determined by many factors, particularly the disintegration and dissolution rates if tablets are given, the pH at the mucosal surfaces, and gastric emptying time.

Salicylate absorption occurs by passive diffusion primarily of nondissociated salicylic acid or acetylsalicylic acid across gastrointestinal membranes and hence is influenced by gastric pH. Even though salicylate is more ionized as the pH is increased, a rise in pH also increases the solubility of salicylate, and the overall effect is to enhance absorption. As a result, there is little meaningful difference between the rates of absorption of sodium salicylate, aspirin, and the numerous buffered preparations of salicylates. The presence of food delays absorption of salicylates.

Rectal absorption of salicylate is usually slower, incomplete, and unreliable; rectal administration is therefore not advisable when high plasma concentrations of the drug are required. Salicylic acid is rapidly absorbed from the intact skin, especially when applied in oily liniments or ointments, and systemic poisoning has occurred from its applica-

tion to large areas of skin. Methyl salicylate is likewise speedily absorbed when applied cutaneously; its gastrointestinal absorption may be delayed many hours, and, therefore, gastric lavage should be performed even in cases of poisoning that are seen late.

When nonionized salicylic acid in the gastric lumen enters mucosal cells, large amounts of salicylate can accumulate because of dissociation to the ionized species at the intracellular pH. As a result, gastric mucosal damage may occur.

Distribution. After absorption, salicylate is distributed throughout most body tissues and most transcellular fluids, primarily by pH-dependent passive processes. Salicylate is actively transported by a low-capacity, saturable system out of the CSF across the choroid plexus. The drug readily crosses the placental barrier.

The volumes of distribution of usual doses of aspirin and sodium salicylate in normal subjects average about 170 ml/kg of body weight; at high therapeutic doses, this volume increases to about 500 ml/kg because of saturation of binding sites on plasma proteins. Ingested aspirin is mainly absorbed as such, but some enters the systemic circulation as salicylic acid, because of hydrolysis by esterases in the gastrointestinal mucosa and the liver. Aspirin can be detected in the plasma only for a short time as a result of hydrolysis in plasma, liver, and erythrocytes; for example, 30 minutes after a dose of 0.65 g, only 27% of the total plasma salicylate is in the acetylated form. As a result, plasma concentrations of aspirin are always low and rarely exceed 20 $\mu\text{g/ml}$ at ordinary therapeutic doses. Methyl salicylate is also rapidly hydrolyzed to salicylic acid, mainly in the liver.

At concentrations encountered clinically, from 80 to 90% of the salicylate is bound to plasma proteins, especially albumin; this fraction declines as plasma concentrations are increased. In addition, hypoalbuminemia, as may occur in rheumatoid arthritis, is associated with a proportionately higher level of free salicylate in the plasma. Salicylate competes with a variety of compounds for plasma protein binding sites; these include thyroxine, triiodothyronine, penicillin, phenytoin, sulfapyrazone, bilirubin, uric acid, and naproxen. Aspirin is bound to a more limited extent; however, it acetylates human plasma albumin *in vivo* by reaction with the ϵ -amino group of lysine; this acetylation may change the binding of drugs to albumin. Hormones, DNA, platelets, and hemoglobin and other proteins are also acetylated.

Biotransformation and Excretion. The biotransformation of salicylate takes place in many tissues, but particularly in the hepatic endoplasmic reticulum and mitochondria. The three chief metabolic products are

salicyluric acid (the glycine conjugate), the ether or phenolic glucuronide, and the ester or acyl glucuronide. In addition, a small fraction is oxidized to gentisic acid (2,5-dihydroxybenzoic acid) and to 2,3-dihydroxybenzoic and 2,3,5-trihydroxybenzoic acids; gentisuric acid, the glycine conjugate of gentisic acid, is also formed.

Salicylates are excreted in the urine as free salicylic acid (10%), salicyluric acid (75%), salicylic phenolic (10%) and acyl (5%) glucuronides, and gentisic acid (<1%). However, excretion of free salicylate is extremely variable and depends upon both the dose and the urinary pH. In alkaline urine, more than 30% of the ingested drug may be eliminated as free salicylate, whereas in acidic urine this may be as low as 2%.

The plasma half-life for aspirin is approximately 15 minutes; that for salicylate is 2 to 3 hours in low doses and about 12 hours at usual antiinflammatory doses. The half-life of salicylate may be as long as 15 to 30 hours at high therapeutic doses or when there is intoxication. This dose-dependent elimination is the result of the limited ability of the liver to form salicyluric acid and the phenolic glucuronide, and a larger proportion of unchanged drug is excreted in the urine at higher doses.

The plasma concentration of salicylate is increased by conditions that decrease glomerular filtration rate or reduce its secretion by the proximal tubule, such as renal disease or the presence of inhibitors that compete for the transport system (e.g., probenecid). Changes in urinary pH also have significant effects on salicylate excretion; for example, the clearance of salicylate is about four times as great at pH 8.0 as at pH 6.0, and it is well above the glomerular filtration rate at pH 8.0. High rates of urine flow decrease tubular reabsorption, whereas the opposite is true in oliguria. The conjugates of salicylic acid with glycine and glucuronic acid do not readily back diffuse across the renal tubular cells. Their excretion, therefore, is both by glomerular filtration and proximal tubular secretion and is not pH dependent.

Preparations, Routes of Administration, and Dosage. The two most commonly used preparations of salicylate for systemic effects are sodium salicylate and aspirin (acetylsalicylic acid).

Sodium salicylate is available in regular or enteric-coated tablets that contain 325 or 650 mg of drug and in an injectable solution for parenteral use. *Aspirin* is available in regular or enteric-coated

tablets ranging from 65 to 975 mg and in suppositories; timed-release tablets are also marketed.

The dose of salicylate depends on the condition being treated. The usual single dose of aspirin in adults is 300 mg to 1.0 g. This may be repeated every 4 hours. More intensive dosage regimens are employed in acute rheumatic fever and rheumatoid arthritis (see below).

The route of administration is nearly always oral. Parenteral administration is rarely necessary. The rectal administration of aspirin suppositories may be necessary in infants or when oral medication is not retained. Salicylates are conveniently taken in tablets or capsules with a full glass of water to minimize gastric irritation. Aspirin is poorly soluble, has many chemical incompatibilities, and should be dispensed only in solid dry form. Timed-release preparations are of limited value, since the half-time for elimination of salicylate is so long, particularly during high-dose therapy. Absorption from enteric-coated tablets is sometimes incomplete, but these formulations may produce less gastrointestinal irritation. Preparations of aspirin containing alkali or buffer are sometimes better tolerated, but alkalinization of the urine, which may occur, can shorten the plasma half-life of salicylates considerably (see above).

Other salicylates that are available for systemic use include *salsalate* (salicylsalicylic acid; DICALCID); it is hydrolyzed to salicylic acid during and after absorption. The drug is available in 500- and 750-mg tablets and 500-mg capsules; the maximal daily dose is 3 g given in 2 to 4 divided doses. *Salicylamide*, which is not metabolized to salicylate *in vivo*, has antipyretic, analgesic, and antiinflammatory effects similar to those of salicylate. It remains available only in certain combination preparations. Sodium thiosalicylate (injection), choline salicylate (oral liquid), and magnesium salicylate (tablets) are also available. A combination of choline and magnesium salicylates (TRILISATE) is formulated to contain 500 mg of salicylate per 5 ml (oral liquid) or 500 to 1000 mg per tablet; 1 to 3 doses per day may be given. The nonacetylated salicylates appear to produce a lower incidence of gastrointestinal ulceration and have less effect on platelet aggregation than does aspirin. Diflunisal is discussed below.

Mesalamine (5-aminosalicylic acid) is a salicylate that is used for its local effects in the treatment of inflammatory bowel disease. The drug is not effective orally because it is poorly absorbed and is inactivated before reaching the lower intestine. It is currently available as a rectal suspension enema (ROWASA) for treatment of mild-to-moderate proctosigmoiditis; formulations that deliver the intact drug to the lower intestine are under investigation (Schroeder *et al.*, 1987). *Sulfasalazine* (salicylazosulfapyridine; AZULFIDINE, AZALINE) contains mesalamine linked covalently to sulfapyridine (see Chapter 45); it is poorly absorbed after oral administration, but it is cleaved to its active components by bacteria in the colon. The drug is of benefit in the treatment of inflammatory bowel disease, principally because of the local actions of mesalamine. Sulfasalazine has also been used in

the treatment of ankylosing spondylitis; sulfapyridine appears to be a component in the treatment.

Methyl salicylate (oil of wintergreen) is used in cutaneous counterirritant form of salves. *Salicylic acid* is used as a keratolytic in ointments, and *salicylic acid* has been used in therapy.

TOXIC EFFECTS

As a result of its availability, salicylate is a cause of intoxication, and sometimes fatal, viewed as a

Hypersensitivity reactions toward response, renal or hepatic thrombinemia, enhance the toxicity. Children are particularly susceptible to relatively small doses, the use of children and illnesses become a syndrome. Many are common and discussed above.

Salicylate Intoxication. Intoxication with the preparation of sodium salicylate in adults, but much more common in one case) has been reported. The lethality is considerably less than that of salicylate as 4 ml (4.7 g) in children.

Symptoms of Intoxication. Intoxication is characterized by hyperventilation, the syndrome of hyperventilation, ringing in the ears, vision, mental confusion, sweating, thirst, and occasional hypotension. A large dose of salicylate produces more pronounced generalized convulsions and marked acidosis, which is usually produced by dehydration often

the treatment of rheumatoid arthritis and ankylosing spondylitis (see Symposium, 1986a, 1988b); sulfapyridine, which is absorbed systemically, appears to be the most important therapeutic component in these conditions.

Methyl salicylate (sweet birch oil, wintergreen oil, *gaultheria* oil, *betula* oil) is employed only for cutaneous counterirritation and is distributed in the form of salves, liniments, and other preparations. *Salicylic acid* is primarily used for local application as a keratolytic agent in plasters, liquids, creams, ointments, and other topical preparations. However, a transdermal patch containing 15% salicylic acid has recently been marketed for systemic therapy.

TOXIC EFFECTS

As a result of their wide use and ready availability, salicylates are frequently the cause of intoxication. Poisoning or serious intoxication often occurs in children and is sometimes fatal. The drug should not be viewed as a harmless household remedy.

Hypersensitivity is also a cause of untoward responses to salicylate. Furthermore, renal or hepatic insufficiency or hypoprothrombinemia or other bleeding disorders enhance the possibility of salicylate toxicity. Children with fever and dehydration are particularly prone to intoxication from relatively small doses of salicylate. In addition, the use of aspirin is contraindicated in children and adolescents with febrile viral illnesses because of the risk of Reye's syndrome. Many of the unwanted effects that are common to the aspirin-like drugs are discussed above.

Salicylate Intoxication. The fatal dose varies with the preparation of salicylate. From 10 to 30 g of sodium salicylate or aspirin has caused death in adults, but much larger amounts (130 g of aspirin, in one case) have been ingested without fatal outcome. The lethal dose of methyl salicylate is considerably less than that of sodium salicylate. As little as 4 ml (4.7 g) of methyl salicylate may be fatal in children.

Symptoms and Signs. Mild chronic salicylate intoxication is termed salicylism. When fully developed, the syndrome includes headache, dizziness, ringing in the ears, difficulty in hearing, dimness of vision, mental confusion, lassitude, drowsiness, sweating, thirst, hyperventilation, nausea, vomiting, and occasionally diarrhea. A more severe degree of salicylate intoxication is characterized by more pronounced CNS disturbances (including generalized convulsions and coma), skin eruptions, and marked alterations in acid-base balance. Fever is usually prominent, especially in children. Dehydration often occurs as a result of hyperpyrexia,

sweating, vomiting, and the loss of water vapor during hyperventilation. Gastrointestinal symptoms are often present; about 50% of individuals with plasma salicylate concentrations of more than 300 $\mu\text{g/ml}$ experience nausea.

A prominent feature of salicylate intoxication is the disturbance in acid-base balance and electrolyte composition of the plasma described above. The most severe metabolic disturbances occur in infants and very young children who become intoxicated as the result of therapeutic overdosage; most of the acidotic patients seen with salicylate intoxication are in this group.

Hemorrhagic phenomena are occasionally seen during salicylate poisoning, the mechanism and significance of which have been discussed. Petechial hemorrhages are a prominent postmortem feature. Thrombocytopenic purpura is a rare complication. While hyperglycemia may occur during salicylate intoxication, hypoglycemia may be a serious consequence of toxicity in young children. It should be seriously considered in any young child with coma, convulsions, or cardiovascular collapse.

Severe toxic encephalopathy may be a prominent feature of salicylate poisoning and may be difficult to differentiate from rheumatic encephalopathy. As poisoning progresses, central stimulation is replaced by increasing depression, stupor, and coma. Cardiovascular collapse and respiratory insufficiency ensue, and terminal asphyxial convulsions and pulmonary edema sometimes appear. Death usually results from respiratory failure after a period of unconsciousness.

Salicylate toxicity in adults may not be readily diagnosed because such patients usually become intoxicated from their therapeutic regimen; there is no history of acute overdosage. Prominent features of toxicity in this group are noncardiogenic pulmonary edema, nonfocal neurological abnormalities, and laboratory findings that include acid-base abnormalities, unexplained ketosis, and a prolonged prothrombin time (Anderson *et al.*, 1976).

Symptoms of poisoning by methyl salicylate differ little from those described for aspirin. Central excitation, intense hyperpnea, and hyperpyrexia are prominent features. The odor of the drug can easily be detected on the breath and in the urine and vomitus. Poisoning by salicylic acid differs only in the increased prominence of gastrointestinal symptoms due to the marked local irritation.

Treatment. Salicylate poisoning represents an acute medical emergency, and death may result despite all recommended procedures. The treatment is largely symptomatic. Salicylate medication is withdrawn as soon as intoxication is suspected. The patient should be hospitalized, particularly in cases of poisoning with methyl salicylate. Blood should be obtained for plasma salicylate determinations and acid-base and electrolyte studies. The salicylate concentration is reasonably well correlated with clinical severity, when corrected for the duration of the intoxication, and is of value in assessing the type of therapy to be instituted. Since absorption of salicylate from the gastrointestinal tract may be delayed for many hours after an overdose, measures to reduce such absorption should

always be employed. These include induction of emesis, gastric lavage, administration of activated charcoal, or a combination of these.

Hyperthermia and dehydration are the immediate threats to life, and the initial therapy must be directed to their correction and to the maintenance of adequate renal function. External sponging with tepid water or alcohol should be provided quickly to any child with very high fever. Adequate amounts of intravenous fluids must be given promptly. The type and amount of solutions to be employed depend upon the interpretation of the laboratory data on acid-base balance. If the patient presents with an acidosis, correction of the low blood pH is essential, especially since acidosis results in a shift of salicylate from plasma into brain and other tissues. Bicarbonate solution should be infused intravenously, if possible, in sufficient quantity to maintain alkaline diuresis. Correction of ketosis and hypoglycemia by administration of glucose is also essential for complete control of the metabolic acidosis; however, the ketosis clears only slowly. If K⁺ deficiency occurs during salicylate intoxication, it should be treated by adding the cation to the intravenous fluids once it has been determined that urine formation is adequate. Plasma transfusion may be beneficial, especially if the shock syndrome intervenes. Hemorrhagic phenomena may necessitate whole-blood transfusion and vitamin K (phytonadione).

Measures to rid the body of salicylate rapidly should be undertaken immediately. Forced diuresis with alkalinizing solution appears to be better than alkali alone; however, this may be dangerous in adults who are prone to develop pulmonary edema. In severe intoxication, hemodialysis is the most effective measure available for the removal of salicylate and for the correction of the electrolyte and acid-base disturbances. Hemodialysis should be considered in patients with salicylate concentrations above 1000 $\mu\text{g/ml}$, in those with severe acid-base disturbances whose clinical condition is deteriorating despite otherwise-appropriate therapy, and in those who have associated serious disease, particularly cardiac, pulmonary, or renal disease. (See Brenner and Simon, 1982; Meredith and Vale, 1986.)

Aspirin Hypersensitivity. Aspirin hypersensitivity or intolerance is discussed above. It is important to recognize this syndrome even though it is rather uncommon, since the administration of aspirin and many other aspirin-like drugs may result in severe and possibly fatal reactions. The non-acetylated salicylates appear to be considerably less apt to produce these reactions as compared with aspirin and other agents. Treatment of such responses does not differ from that ordinarily employed in acute anaphylactic reactions. Epinephrine is the drug of choice and usually controls angioedema and urticaria without difficulty.

THERAPEUTIC USES

There are many systemic and a few local uses of the salicylates. Several are based on

tradition and empirical results rather than on a clear understanding of the mechanism of therapeutic benefit.

Systemic Uses. *Antipyresis.* Antipyretic therapy is reserved for patients in whom fever in itself may be deleterious, and for those who experience considerable relief when a fever is lowered. Little is known about the relationship between fever and the acceleration of inflammatory or immune processes; it may at times be a protective physiological mechanism. The course of the patient's illness may be obscured by the relief of symptoms and the reduction of fever from the use of antipyretic drugs. The antipyretic dose of salicylate for adults is 325 to 650 mg orally every 4 hours; for children, 50 to 75 mg/kg per day is given in four to six divided doses, not to exceed a total daily dose of 3.6 g.

Analgesia. Salicylate is valuable for the nonspecific relief of certain types of pain, for example, headache, arthritis, dysmenorrhea, neuralgia, and myalgia. For this purpose, it is prescribed in the same doses and manner as for antipyresis.

Acute Rheumatic Fever. In this disease, the salicylates suppress the acute exudative inflammatory process but do not affect the duration or progression of the disease or the later phases of granulomatous inflammation or scar formation. Nevertheless, if a patient has severe carditis and heart failure, the nonspecific antiinflammatory effect of salicylates and particularly of adrenocorticosteroids may be invaluable in reducing the burden upon the heart.

For maximal suppression of rheumatic inflammation, doses that provide a plasma salicylate concentration of 150 to 300 $\mu\text{g/ml}$ should be maintained, but polyarthritides and fever usually respond to smaller amounts. For adults, a total daily dosage of 5 to 8 g. given at intervals in 1-g amounts, usually suffices. Children are given 100 mg/kg per day, in divided portions every 4 to 6 hours, for up to 1 week; the dose is then reduced in stepwise fashion at weekly intervals to 60 to 75 mg/kg per day and maintained as long as necessary. Anorexia, tinnitus, nausea, and vomiting are common during the first 3 or 4 days of therapy, but tend to subside despite continuation of medication. Ordinarily, full doses are continued until at least 2 weeks after the patient is asymptomatic and all evidence of active inflammation has disappeared. The drug is then gradually discontinued over a period of 7 to 10 days. If symptoms and signs of the disease reappear, salicylate therapy is reinstituted. Therapy with glucocorticoids does not yield overall results superior to those obtained with the salicylates; salicylate and glucocorticoids are additive in their effects. If carditis is not evident, salicylates and not steroids should be used. However, if acute severe carditis is present, most investigators believe adrenocorticosteroids should be given instead of salicylates, at least initially.

Rheumatoid Arthritis. Despite the development of the newer antiinflammatory agents, salicylates are still regarded as the standard with which other drugs should be compared for the treatment of

rheumatoid arthritis that allows normal function. There is improvement in overall well-being. Satisfaction in joint function. Damage to joints in rheumatoid arthritis reduces the impact of delaying treatment. Analgesics can be substituted for antiinflammatory drugs for long-term treatment of rheumatoid disease. Analgesics used for pain are advised, but

The majority of patients with rheumatoid arthritis can be controlled with low-dose aspirin. In other aspirin-resistant patients, low-dose corticosteroids require therapeutic doses of aspirin. In patients with severe disease, low-dose corticosteroids require therapeutic doses of aspirin.

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rheumatoid arthritis. In addition to the analgesia it allows more effective therapeutic exercises, is improvement in appetite and a feeling of being. Salicylates also reduce the inflammation in joint tissues and surrounding structures. Damage to joints is the most difficult aspect of rheumatoid arthritis to manage, and any agent that reduces the inflammation is important in lessening or delaying the development of crippling. Salicylates can be shown to produce objectively measurable antiinflammatory changes when given in large doses for long periods to patients with active rheumatoid disease. Large doses of salicylates, such as those used for rheumatic fever (4 to 6 g daily), are advised, but some patients respond well to less.

The majority of patients with rheumatoid arthritis can be controlled with salicylates alone or with other aspirin-like antiinflammatory agents. Some require therapy with more toxic drugs, such as gold salts, hydroxychloroquine, penicillamine, adrenocorticosteroids, or immunosuppressive agents.

Other Uses. Because of the potent and long-lasting effect of low doses of aspirin on platelet function, this drug is used in the treatment or prophylaxis of diseases associated with platelet hyperaggregability, such as coronary artery disease and postoperative deep-vein thrombosis (*see* Chapter 55). The effectiveness of such therapy appears to depend upon blockade of TXA_2 synthesis by platelets without preventing production of PGI_2 by endothelial cells (*see* Chapters 24 and 55). Although the optimal dosage has not been established, the frequency of beneficial effects appear to be greater when the dose of aspirin is 325 mg per day or lower. In the largest study to date, the ingestion of 125 mg of aspirin every other day reduced the incidence of myocardial infarction in male physicians more than 40%; no effect was detected on the incidence of stroke (Steering Committee of the Physicians' Health Study Research Group, 1989).

A relative excess of TXA_2 over PGI_2 has been implicated in the genesis of preeclampsia and hypertension induced by pregnancy (*see* Lubbe, 1987). The administration of 60 or 100 mg of aspirin per day to pregnant women who have a high risk of developing hypertension reduces the formation of thromboxane A_2 without changing the production of PGI_2 and may lower the incidence of preeclampsia (Benigni *et al.*, 1989; Schiff *et al.*, 1989).

Relationship of Plasma Salicylate Concentration to Therapeutic Effect and Toxicity. For optimal antiinflammatory effect for patients with rheumatic diseases, plasma salicylate concentrations of 150 to 300 $\mu\text{g/ml}$ are required. In this range, the clearance of the drug is nearly constant (despite the fact that saturation of metabolic capacity is approached) because the fraction of drug that is free and thus available for metabolism or excretion increases as binding sites on plasma proteins are saturated. The total concentration of salicylate in plasma is thus a relatively linear function of dose. It is important to individualize the total dose of aspirin, especially because the range of plasma salicylate concentrations needed for optimal antiinflammatory effects may overlap that at which tinnitus is noted. Tinnitus may be a reliable index of therapeutic plasma

concentration in patients with normal hearing, but obviously not in those with a preexisting hearing loss. Hyperventilation generally occurs at concentrations greater than 350 $\mu\text{g/ml}$, and other signs of intoxication, such as acidosis, at concentrations greater than 460 $\mu\text{g/ml}$. Single analgesic-antipyretic doses of salicylate usually yield plasma concentrations below 60 $\mu\text{g/ml}$.

The plasma concentration of salicylate is generally little affected by other drugs, but concurrent administration of aspirin lowers the concentrations of indomethacin, naproxen, and fenoprofen, at least in part by displacement from plasma proteins. Important adverse interactions of aspirin with warfarin and methotrexate are mentioned above. Other interactions of aspirin include the antagonism of spironolactone-induced natriuresis and the blockade of the active transport of penicillin from CSF to blood.

Local Uses. Salicylic acid is applied topically as a keratolytic agent. In combination with benzoic acid, it is often prescribed for epidermophytosis. Salicylic acid is also employed as a wart and corn remover (10 to 20% in collodion).

Methyl salicylate is reserved for external use as a counterirritant. It is employed for painful muscles or joints and distributed in an ointment, liniment, or other preparation. Absorption of methyl salicylate can occur through the skin, and death has resulted from systemic poisoning from the local misapplication of the drug. It is a common pediatric poison, and its use should be strongly discouraged. It is also used as a flavoring agent.

DIFLUNISAL

Diflunisal is a difluorophenyl derivative of salicylic acid (*see* Table 26-1); it is not converted to salicylic acid *in vivo*. Diflunisal is more potent than aspirin in antiinflammatory tests in animals and appears to be a competitive inhibitor of cyclooxygenase. However, it is largely devoid of antipyretic effects, perhaps because of poor penetration into the CNS. The drug has been used primarily as an analgesic in the treatment of osteoarthritis and musculoskeletal strains or sprains; in these circumstances it is about three to four times more potent than aspirin. Diflunisal does not produce auditory side effects and appears to cause fewer and less intense gastrointestinal and antiplatelet effects than does aspirin.

Diflunisal is almost completely absorbed after oral administration, and peak concentrations occur in plasma within 2 to 3 hours. It is extensively bound to plasma albumin (99%). Diflunisal appears in the milk of lactating women; its penetration into the CNS is uncertain. About 90% of the drug is excreted as glucuronides, and its rate of elimination is dependent upon dosage. At the usual analgesic dose (500 to 750 mg per day) the plasma half-life ranges between 8 and 12 hours. (For reviews, *see* Brogden *et al.*, 1980; Davies, 1983; van Winzum *et al.*, in Symposium, 1983a.)

Diflunisal (DOLOBID) is marketed in 250- and 500-mg tablets. For mild-to-moderate pain, the

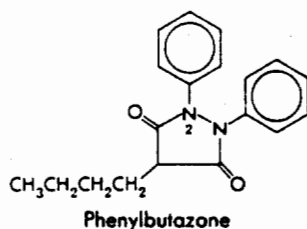
usual initial dose is 500 to 1000 mg, followed by 250 to 500 mg every 8 to 12 hours. For rheumatoid arthritis or osteoarthritis, 250 to 500 mg is administered twice daily; maintenance dosage should not exceed 1.5 g per day.

PYRAZOLON DERIVATIVES

This group of drugs includes phenylbutazone, oxyphenbutazone, antipyrine, aminopyrine, dipyrone, and a more recent addition, apazone (azapropazone). With the exception of apazone, these drugs have been in clinical use for many years; although not a first-line drug, phenylbutazone is the most important from the therapeutic viewpoint, while antipyrine, dipyrone, and aminopyrine are seldom used today. Apazone is not yet available in the United States.

PHENYLBUTAZONE

Phenylbutazone was introduced in 1949 for the treatment of rheumatoid arthritis and allied disorders. Although it is an effective antiinflammatory agent, serious toxicity limits its use in long-term therapy. Its structural formula is as follows:



Pharmacological Properties. The antiinflammatory effects of phenylbutazone are similar to those of the salicylates, but its toxicity differs significantly. Like aminopyrine, phenylbutazone can cause agranulocytosis. The pharmacology and toxicology of phenylbutazone and its metabolites and congeners have been reviewed in a symposium (Symposium, 1983a) and by Schuster and associates (Rainsford, 1985a).

Antiinflammatory Effects. Phenylbutazone has prominent antiinflammatory effects, and its frequent use to enhance the performance of race horses is well known. Somewhat similar effects are demonstrable in patients with rheumatoid arthritis and related disorders.

Antipyretic and Analgesic Effects. The antipyretic effect of phenylbutazone has been little studied in man. For pain of nonrheumatic origin, its analgesic efficacy is inferior to that of salicylates. Because of its toxicity, phenylbutazone should not be used routinely as an analgesic or antipyretic.

Uricosuric Effect. In doses of about 600 mg per day, phenylbutazone has a mild uricosuric effect, probably attributable to one of its metabolites that decreases tubular reabsorption of uric acid. Low concentrations of the drug inhibit tubular secretion of uric acid and cause retention of urate. A congener, sulfinpyrazone, is a much more effective uricosuric agent and is useful for the treatment of chronic gout (see below and Chapter 30).

Effects on Water and Electrolytes. Phenylbutazone causes significant retention of Na^+ and chloride, accompanied by a reduction in urine volume; edema may result. The excretion of K^+ is not changed. Plasma volume frequently increases by as much as 50%, and, as a result, cardiac decompensation and acute pulmonary edema have occurred in patients given the drug.

Other Effects. Phenylbutazone reduces the uptake of iodine by the thyroid gland, apparently secondary to inhibition of biosynthesis of organic iodine compounds. Goiter and myxedema may occasionally result from this effect.

Pharmacokinetics and Metabolism. Phenylbutazone is rapidly and completely absorbed from the gastrointestinal tract or the rectum, and the peak concentration in plasma is reached in 2 hours. After therapeutic doses, more than 98% of phenylbutazone is bound to plasma proteins. The half-life of phenylbutazone in plasma is very long—50 to 65 hours. The drug penetrates into the synovial spaces and reaches a concentration about one half of that in the plasma; significant concentrations may persist in the joints for up to 3 weeks after treatment is discontinued.

Phenylbutazone undergoes extensive metabolic transformation in man. The most significant primary reactions involve glucuronidation and hydroxylation of the phenyl rings or the butyl side chain. The conjugates are excreted in the urine and represent the bulk of the excreted drug. Oxyphenbutazone, a metabolite of phenylbutazone, has antirheumatic and Na^+ -retaining activities similar to those of the parent drug. Oxyphenbutazone is also extensively bound to plasma proteins and has a half-life in plasma of several days. It accumulates significantly during long-term administration of phenylbutazone and contributes to the pharmacological and toxic effects of the parent drug. Only a trace of unchanged phenylbutazone is excreted in the urine. Oxyphenbutazone is excreted mainly as the O-glucuronide.

Drug Interactions. Other antiinflammatory agents, oral anticoagulant drugs, oral hypoglycemics, sulfonamides, and other drugs may be displaced from binding to plasma proteins by phenylbutazone. The net result depends upon the drug and its disposition after being displaced. The well-documented increased risk of bleeding associated

with concurrent use in part importantly, phenylbutazone displacement of the hormone component tests.

Phenylbutazone inhibits microsomal enzyme function of other microsomal systems, such as the effect of insulin.

Toxic Effects. Phenylbutazone is noted in 10 to 40% of patients have to be discontinued because of vomiting, epigastric pain, diarrhea, vertigo, hematuria, and edema.

More serious toxic effects include peptic ulcer (or perforation), serum-sickness-like reaction, nephritis, aplastic anemia, and deaths have occurred.

When phenylbutazone should be closely monitored and examined frequently. The drug should be discontinued if more than 1 week of disturbing side effects are observed. The physician should be told to the physician if other oral lesions, weight gain, or toxic effects are noted in patients or hepatic dysfunction, blood dyscrasia. The toxic effect is more pronounced in elderly persons. Its use is not recommended.

Preparations. Phenylbutazone is available in 100-mg capsules for oral administration. In brief periods of higher doses on a case may be substituted by doses as low as 100 mg should be used to avoid irritation.

Therapeutic Use. Phenylbutazone is not used for any condition except for the treatment of rheumatoid arthritis.

seborrhoeic dermatitis in addition to the povidone-iodine shampoo. On this regimen, her scalp and nails have remained free of *Staph. aureus*.

Discussion

Everybody carries bacteria in varying amounts in their hair, and in many instances large numbers of organisms are of a virulent type. Nevertheless, epidemics of wound infections do not often occur although carriers are present in the operating-theatre. This indicates that host factors may also be important. In the first epidemic several patients had reduced host resistance because of a poor blood-supply to the area involved. Also the surgical technique of the doctor who was a carrier often produced wounds with excessive amounts of necrotic material and dead space left behind. Therefore, local host factors which favoured the development of wound infection were involved in the first outbreak. In the second outbreak all the infections occurred in patients who were either on dialysis or had recently had transplants, all were people with extremely poor host resistance. Many were on immunosuppressive drugs. Thus it seems that simple carriage of *Staph. aureus* in the hair is not sufficient to establish clinical sepsis, there must also be poor host resistance (on a local or systemic basis).

In our experience routine sampling of people in critical areas of the hospital is of no significant value, and broad-scale sampling should be reserved for outbreaks when they occur. From our experience of the present two outbreaks we believe that during epidemics of wound infection cultures should be taken of the hair and subungual spaces as well as the nose and throat.

Since these outbreaks the Rodac contact-plate technique has been used to determine the frequency of hair carriage in ten people. The average pre-shampoo counts were 323 organisms. Washing the hair with any of the antibacterial shampoos considerably reduced the count. However, the general experience both here and in other hospitals has been that povidone-iodine shampoo gives the best reduction.

It is also important to reduce the amount of shedding from the hair and scalp in the operating-theatre, and this is best achieved by covering the hair (preferably with a hood) so that the hair and scalp are not exposed during the operation.

The infections which resulted from hair carriage are probably unusual and require poor host conditions and delivery of virulent organisms into the wound. Since the present study, several people who carried virulent staphylococci in their hair have been identified and yet there were no associated epidemics of wound infections in patients under their care.

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INFLUENCE OF ACETYLSALICYLIC ACID, AN INHIBITOR OF PROSTAGLANDIN SYNTHESIS, ON THE DURATION OF HUMAN GESTATION AND LABOUR

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Summary A retrospective survey of 103 patients taking high-dose acetylsalicylic acid for at least the last 6 months of pregnancy has been carried out and comparisons made with control populations. Aspirin administration was associated with a highly significant increase in the average length of gestation, in the frequency of postmaturity, and in the mean duration of spontaneous labour. These observations are compatible with the known capacity of aspirin to inhibit the synthesis of prostaglandins, and suggest that endogenous prostaglandins are important regulators of the duration of human gestation and labour. The possibility is raised that aberrations in prostaglandin metabolism could be responsible for certain instances of postmaturity or prolonged labour.

Introduction

VARIOUS prostaglandins, notably P.G.F₂ and P.G.E₂, are of clinical interest in obstetrics because of their ability to cause contractions of the gravid uterus.¹ These prostaglandins can stimulate mid-trimester abortions or induce labour near term.²⁻⁴ Although prostaglandins appear in increased levels in maternal blood and in amniotic fluid during spontaneous labour or abortion,⁵ the physiological significance of these endogenous substances in the regulation of gestation and labour is not clear.

Vane's group and others have demonstrated that acetylsalicylic acid and aspirin-like compounds are potent inhibitors of prostaglandin synthesis,⁶⁻¹⁰ and Waltman et al. have found that aspirin and indomethacin prolong the time course of abortions induced with hypertonic saline.^{11,12} We felt it would be of interest to study gestation in women who had taken aspirin in high doses for a long time. A prospective investigation seemed to be precluded for ethical reasons, in view of the potential dangers of aspirin administration during pregnancy, especially bleeding,¹³ so a retrospective technique was used. The investigation revealed a significant prolongation of the duration of gestation and of labour in patients receiving aspirin, and a striking increase in the frequency of postmaturity.

Patients and Methods

Obstetrical records of patients delivered at the New York Lying-In Hospital between 1951 and 1971 were reviewed. Patients delivered by caesarean section or who had had induction or augmentation of labour were ex-

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cluded. Therapeutic dosage of aspirin was arbitrarily defined as a daily consumption greater than 3250 mg. (50 grains) for at least the last 6 months of gestation. We found 103 patients who met these criteria (group I): most of them had rheumatoid arthritis, non-specific collagen disease, or degenerative musculoskeletal disease and were found by review of charts coded for these diagnoses during pregnancy. Two control groups were chosen in an unselected manner from the same 20-year period. The first control group (group II) consisted of 52 patients with rheumatoid arthritis, non-specific collagen disease, or degenerative musculoskeletal disease during gestation who were not taking aspirin or other compounds known to affect prostaglandin metabolism; some of these patients were taking corticosteroids. The second control group (group III) contained 50 pregnant women without known disease who also were not taking therapeutic doses of aspirin or related drugs. All data were drawn directly from antenatal and delivery records, and were analysed for statistical significance using the *t* or Chi-square tests with the aid of an Olivetti Underwood 101 computer. A *P* value of <0.05 was considered significant.

Results

The data of major interest are summarised in the table. There was no statistically significant difference in the mean age, parity, or gravidity of the three groups.

Length of gestation was calculated from the best estimate or actual date of the patient's last menstrual

COMPARISONS IN THE THREE STUDY GROUPS (MEAN \pm S.D.)

Variable	Group I (103)	Group II (52)	Group III (50)
Age (yr.) ..	26.0 \pm 5.68	26.7 \pm 5.05	26.3 \pm 5.18
Gravidity ..	2.55 \pm 1.69	2.69 \pm 1.34	2.52 \pm 1.60
Parity ..	1.37 \pm 1.52	1.50 \pm 1.43	1.62 \pm 1.40
Length of gestation (days) ..	286.1 \pm 13.3	275.2 \pm 10.6	278.6 \pm 6.91
Length of labour (hr.) ..	12.1 \pm 10.6	7.30 \pm 4.11	6.96 \pm 4.96
Birth-weight (g.) ..	3077 \pm 597	2972 \pm 538	3379 \pm 460
Blood-loss estimated (ml.) ..	340 \pm 155	244 \pm 114	235 \pm 97

* Mean different ($P < 0.05$) from other two groups. Otherwise, differences between groups were not significant at this level.

period to the date of delivery. Patients taking aspirin (group I) had an average period of gestation over 1 week longer than either control group, and these differences are highly significant ($P < 0.025$). The control groups did not differ significantly from each other in this respect.

The change in the mean length of gestation concomitant with aspirin administration is associated with a striking increase in gestations lasting more than 42 weeks (at least 15 days post-mature). 42% of the aspirin group had gestations of more than 42 weeks, while only 3% of the combined control groups demonstrated this. This difference is very significant ($P < 0.001$). We could detect no trend toward diminishing rates of preterm deliveries in the aspirin group compared with the controls.

As shown in the table, patients taking aspirin (group I) had a much longer average length of labour than either of the other two groups. For these

calculations, patients with premature rupture of membranes were excluded, and labour was considered to have started when contractions occurred regularly every 5 minutes if noted in the record; alternatively, the time of onset of labour indicated by the obstetrician was used. The average duration of labour in group-I patients was 12 hours and 7 minutes, approximately 5 hours greater than either control group. This difference is highly significant ($P < 0.005$), while groups II and III did not differ significantly from each other. In groups II and III no labour lasted longer than 24 hours. In group I, 18 patients had labours lasting longer than 24 hours ($P = 0.001$).

The birth-weights of infants born to group-I mothers averaged 3077 g., and this was not significantly different from the average 2972 g. birth-weight of infants born to group-II mothers, who had similar diseases but took no aspirin. However, the normal mothers in group III had infants whose average birth-weight was 3379 g., and this was significantly heavier ($P < 0.05$) than the average weight in either groups I or II. This result, in which a group of healthy women had rather larger newborns than women many of whom had serious systemic diseases, is perhaps not surprising on intuitive grounds; the absence of an aspirin effect on this variable is noteworthy.

The table also indicates the average estimated blood-loss during delivery for each group. Patients taking aspirin had an average blood-loss of 340 ml., which was about 100 ml. more than either control group. This difference was significant ($P < 0.025$). Massive blood-loss of 1000 ml. or more occurred only once in group I, and not at all in the other groups. Uterine atony requiring transfusion, uterine packing, and/or hysterectomy was not observed in any patient in this study.

Discussion

Since aspirin is a potent inhibitor of prostaglandin synthesis⁶⁻¹⁰ and since the prostaglandins are endogenous compounds capable of causing uterine contraction, we anticipated that if the prostaglandins were important factors contributing to determining the duration of gestation and labour, an effect of aspirin administration might be evident in a retrospective study of the kind presented here.

A substantial group of patients taking high-dose acetylsalicylic acid during pregnancy (group I) were compared with control groups (II and III) which were similar to each other and to the aspirin group patients in gravidity, parity, and age. One of the control groups (II) had diseases similar to those in the aspirin-taking population. Our results support the view that prostaglandin metabolism may be an important determinant of the timing of the onset of spontaneous labour and of its duration.

Patients in group I had gestations lasting over 1 week longer, on the average, than the controls. Interestingly, there was a striking increase in the frequency of postmaturity (defined here as gestations over 42 weeks) in patients taking high-dose aspirin.

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These observations not only immediately suggest that prostaglandins are involved in the timing of onset of labour, but also the possibility that certain cases of postmaturity may be related to abnormalities of prostaglandin metabolism. Postmaturity is of substantial concern to the obstetrician, particularly in view of its association with a variety of increased fetal risks,¹² yet in most cases its aetiology is unknown. We found no tendency towards reduced rates of prematurity in the patients taking aspirin. However, we do not suggest that such an effect might not become apparent in a larger series, nor that the possible future development of safe "anti-prostaglandins" could not prove to be important in reducing the incidence of prematurity.

Patients taking aspirin had labours which averaged close to 70% longer than those in the control populations. In the aspirin group, 18 patients had labours lasting longer than 24 hours; there were none this long in the controls. This is compatible with the hypothesis that endogenous prostaglandins are significant determinants of the duration of labour, presumably mediated through some effect on the contractility of uterine muscle. The intriguing possibility that abnormalities of prostaglandin metabolism may be responsible for certain cases of desultory or prolonged labour can therefore be suggested.

Patients taking high-dose aspirin lost significantly more blood at delivery than control individuals. This result is not surprising, since various effects of aspirin on platelets and clotting activity are well documented.⁸ Post-partum haemostasis is achieved by intense myometrial contractility as well as the blood-coagulation mechanism. One cannot say whether some diminution in the uterine tone post partum of group-I patients compared to controls, mediated through alterations of prostaglandin metabolism by aspirin, could have been present and contributed to the greater blood-loss in group I. In this study, major degrees of post-partum uterine atony were not noted in the patients taking aspirin.

Studies we have in progress will continue to investigate the influence of aspirin in human gestation, and in particular to further characterise the effects of this drug on the progeny.

We thank Prof. Fritz Fuchs and Miss J. McDonald for their assistance.

Requests for reprints should be addressed to R. B. L.

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DARK-ADAPTATION TESTING FOR DIAGNOSIS OF SUBCLINICAL VITAMIN-A DEFICIENCY AND EVALUATION OF THERAPY*

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Summary Thirteen patients with chronic small-intestinal disease without overt evidence of nutritional deficit were found to have abnormal dark-adaptation tests. Ten patients were receiving 3500 to 10,000 units of vitamin A daily in oral multivitamin preparations. Dark adaptation did not change in five patients after a week of oral vitamin-A therapy (25,000 units per day). Normal dark adaptation was restored in two of four patients after a single dose of parenteral vitamin A (50,000 units) and in five of seven patients after 30 days of oral therapy (50,000 units per day). The high frequency of subclinical vitamin-A deficiency found in this group suggests that occult nutritional deficiency may be more prevalent in patients with intestinal disease than is commonly appreciated. Routine vitamin supplementation cannot be relied upon to prevent the functional deficit demonstrated in this study.

Introduction

NUTRITIONAL status, in terms of function, is difficult to assess and even more difficult to measure. Clinical signs and symptoms of hypovitaminosis cannot be used for early detection of deficiency. Moreover, biochemical assays of serum or urine vitamin levels are uncertain indicators of deficiency in individual cases. In patients with chronic disease, surveillance for vitamin deficiency is especially important, because restricted diets, impaired absorption, and overutilisation can lead to a slow depletion of vitamin stores. Even when routine vitamin supplementation is given, the adequacy of this therapy is uncertain.

We have evaluated the adequacy of vitamin status in patients with treated chronic small-intestinal disease. Vitamin A was studied because vitamin-A-dependent visual function is a very sensitive index of the adequacy of vitamin-A status and can be measured by dark-adaptation tests.¹

Patients and Methods

Six patients with regional enteritis, six patients with treated coeliac sprue, and one patient with jejunal diverticulosis without demonstrable bacterial overgrowth were selected for study from the patients of the University of Chicago Hospitals and Clinics. Nine were females and four were males, with ages ranging from 21 to 75. All

* Presented in part by R. R. at the annual meeting of the American Society for Clinical Nutrition held in Atlantic City, New Jersey, in May, 1973.

† Dr Krill died on Aug. 12, 1972.

FETAL EFFECTS OF REGULAR SALICYLATE INGESTION IN PREGNANCY

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Summary The babies of 144 mothers who took salicylates regularly in pregnancy are described. These babies had a significantly reduced birth-weight compared with controls; many of them had a raised cord-blood salicylate level but had no clinical evidence of bleeding or hypoglycaemia. Their perinatal mortality was increased, but the incidence of congenital anomalies was not significantly raised.

Introduction

It has been suggested that salicylates may be teratogenic. Proof of this would be very important because salicylate-containing preparations are freely available and widely used. The suspicion of teratogenicity was raised in two large surveys^{1,2} of the drugs taken during pregnancy by women who gave birth to babies with congenital anomalies. The proportion of mothers who had taken salicylates in the early months of pregnancy was higher for babies with anomalies than for the controls. Salicylates are teratogenic in laboratory animals, usually producing vascular or skeletal anomalies. They have also been associated with reduced fetal weight, fetal resorptions, and haemorrhage.³ In smaller dosage, salicylates given to pregnant rats produce progeny with impaired learning ability.⁴

Salicylates cross freely into the fetal circulation, so if these drugs are taken by the mother during labour, raised salicylate levels would be expected in the baby. Salicylates have many pharmacological actions: they affect glucose metabolism and platelet aggregation, and it might be anticipated therefore that their presence in the newborn would be associated with some clinical effect.

This study was initiated to attempt to answer the question of whether salicylates were teratogenic and whether a raised level of blood salicylate was of any clinical significance in the neonatal period.

Methods

The method of identifying the 144 mothers is described in the preceding paper.⁵ Maternal blood taken at the time of delivery and maternal and cord blood salicylate levels were measured using a colorimetric method.⁶ 'Dextrostix'

tests were done by the nursery staff on the babies six hours after delivery or before the first feed whichever was earlier. The babies were examined by one of us and were divided into group 1 (64) if their mothers took salicylate daily and group 2 (82) if their mothers took it at least once per week. Mothers in group 1 were matched with controls for age, parity, gravity, ethnic group, and social class.

Results

Table I shows the major congenital anomalies in the two groups and the controls. The frequency of major congenital anomalies over the two-year period of the study in all women attending the antenatal clinics at this hospital was 2.4%.

Table II shows the blood-salicylate levels of the mothers at the time of delivery and the cord-blood levels in the babies. This was not done in all the mothers, and maternal blood was not always collected immediately after delivery but always while the mother was still in the labour ward. It was not possible, therefore, to compare maternal-blood and cord-blood levels directly, but, as expected, where the maternal-blood level was high so was the cord-blood level.

The babies tended to be of low birth-weight (table III). The birth-weights in the two groups and the controls were corrected for gestation and being first-born using Tanner charts and for smoking by adding

TABLE I—MAJOR CONGENITAL ANOMALIES

Group	No.	Anomalies	No. of anomalies
1	64	Ventricular septal defect (v.s.d.) Holt-Oram syndrome	2
2	82	Hypoplastic left-heart syndrome Diaphragmatic hernia Perforation of large bowel—3 v.s.d.s Atrial septal defect	4
Controls	64	v.s.d.	1

TABLE II—SERUM-SALICYLATE LEVELS

—	No.	Serum-salicylate (mg. 100 ml.)				
		0-1	1.1-3	3.1-5	5.1-7	7.1-9
Mothers . .	81	13	60	7	1	0
Babies . .	76	21	45	5	2	3

TABLE III—MEAN CORRECTED BIRTH-WEIGHTS (g.)

—	Group 1	Group 2	Controls
Present pregnancies . .	3283*	3372	3502

Mean for controls—mean for groups 1-2 = 170 g.

* Significantly lower than mean control birth-weight ($P < 0.005$).

TABLE IV—BIRTH-WEIGHTS OF LIVEBORN GROUP 1 BABIES RELATED TO DURATION OF SALICYLATE TAKING

Duration of salicylate taking (yr.)	Males		Females	
	Mean birth-weight (g.)	No.	Mean birth-weight (g.)	No.
0-4	3377	8	3124	12
5-9	3286	8	2900	5
10-14	3151	9	2891	9
15-	2901	5	2910	2

DR COLLINS, DR TURNER: REFERENCES

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TABLE V—F

Stillbirth-rate* Neonatal-mortality Perinatal-mortality

* Fetal deaths at than 400 g.)
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TABLE VI—STILLBIRTHS IN PRESENT PREGNANCIES

No.	Sex	Gestation (wk.)	Birth-weight (g.)	Maternal age (yr.)	Salicylate consumption (yr.)	Pregnancy complications
Group 1:						
1	F	37	2305	30	14	Nil
2	M	39	3050 (macerated)	35	17	Anæmia (Hb 8.9 g./100 ml.)
3	F	36	2570	38	20	A.P.H., P.P.H. (blood-loss 3 l.)
4	F	36	2490 (macerated)	35	10	Nil
Group 2:						
1	M	29	1920	38	12	A.P.H. (blood-loss 3.5 l.)

A.P.H. = Ante-partum hæmorrhage. P.P.H. = Post-partum hæmorrhage.

180 g.⁷ Multiple births were excluded. Table IV shows that the birth-weight decreased with increasing duration of salicylate taking. Clinical examination of these babies showed that many of them had features of dysmaturity such as cracked skin and lack of subcutaneous fat. In spite of this, the dextrostix value was below 20 mg. in only 1 baby. This baby weighed 1580 g. at term and developed clinical hypoglycæmia in the first twenty-four hours and then clinical hypocalcæmia at ten days of age.

None of the 146 babies developed any serious hæmorrhagic features although 1 had a few scattered petechiæ and 1 had a cephalhæmatoma. Some of the babies became jaundiced but none so badly that phototherapy or exchange transfusion was required. Some of the babies appeared lethargic with feeding, but all were discharged home on the same day as their mother, except the 1 with hypoglycæmia and 3 with congenital anomalies.

Table V shows the fetal wastage in the observed pregnancies and in the mother's previous pregnancies

TABLE V—FETAL WASTAGE: PRESENT + PAST PREGNANCIES

	Group 1	Group 2	Controls
Stillbirth-rate*	58 ‡	24	12
Neonatal-mortality rate*	31	39	16
Perinatal-mortality rate‡	87 ‡	62	27

* Fetal deaths at 20 wk. gestation or later (or birth-weight greater than 400 g.) per 1000 total births.

† Deaths within first 28 days of life per 1000 live births.

‡ Stillbirths + neonatal deaths per 1000 total births.

§ Significantly increased over controls ($P < 0.01$ and < 0.005 , respectively).

during which she had taken salicylate. The clinical features of the pregnancies of the 5 women who had a stillbirth are shown in table VI. The 3 neonatal deaths were due to congenital abnormalities.

Discussion

The results of the survey were unexpected. We had thought that we might detect teratogenicity, neonatal hypoglycæmia, and bleeding—but what we found was an increased stillbirth-rate and a significantly reduced birth-weight in babies who were otherwise clinically unremarkable.

The frequency of major anomalies in the salicylate group was not significantly raised. There were more anomalies in the group of women who took salicylates intermittently rather than constantly, suggesting that if there is any teratogenic effect it may be more related to fluctuating levels of salicylate than a constantly

elevated level. The teratogenicity suggested in the retrospective studies was significant in respect to abnormalities of the central nervous system, but no such lesions were found in our study. It may well be, as was suggested by Richards¹ and by Nelson and Forfar,² that the teratogenicity related to the illness for which salicylates were taken than a direct effect of the salicylates themselves.

Some babies were born with a raised cord-blood level of salicylate; this was not associated with hypoglycæmia, bleeding, or any other obvious clinical disturbance. Others have found that platelet aggregation is inhibited and have reported babies with serious bleeding manifestations associated with maternal salicylate ingestion.⁸ This may happen, but the evidence of our series suggests that it must be uncommon.

The birth-weights of babies born to mothers taking salicylates were lower than those of matched controls even after correction for maternal smoking. The birth-weight also decreased with increasing length of time of taking salicylates. This suggests that it may not be solely an effect of salicylates on fetal growth but rather a cumulative secondary effect from some maternal factor. This factor might be renal in origin, although no mother was in renal failure and the incidence of renal-tract infection and toxæmia was not significantly raised.

The increased stillbirth-rate was very significant in comparison with the control group and with the rate for the hospital population. This was not solely related to ante-partum hæmorrhage and did not correlate with pregnancies going beyond term or the fetus being excessively small, but it did happen in the older mothers who had been taking salicylates for a number of years.

Our findings do not support the suggestion that salicylates are teratogenic, but they do suggest that chronic salicylate ingestion is associated with an increase in perinatal mortality and with decreased intrauterine growth.

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Low-dose aspirin in the prevention of preeclampsia and fetal growth retardation: Rationale, mechanisms, and clinical trials

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Preeclampsia is characterized by a functional imbalance between vascular prostacyclin and thromboxane A_2 production. On the basis of the hypothesis that preeclampsia is at least partially caused by an increase in thromboxane A_2 , some studies attempted to correct this pathologic condition by pharmacologic manipulation with low-dose aspirin. The current literature suggests that the use of low-dose aspirin during pregnancy is safe with regard to congenital anomalies and fetal, neonatal, and maternal cardiovascular physiologic state and hemostasis. Aspirin at least partially corrects the pathologic increase in angiotensin II sensitivity that precedes the clinical development of preeclampsia. In addition, some clinical trials have demonstrated that low-dose aspirin is effective in reducing the incidence of preeclampsia and/or fetal growth retardation in selected high-risk women. Currently, large clinical trials are in progress to evaluate the effectiveness and side effects of the use of low-dose aspirin in preventing preeclampsia and/or fetal growth retardation. Until these studies have been completed, it will remain unclear whether antiplatelet therapy, such as low-dose aspirin, should be adopted for the prevention of either preeclampsia or fetal growth retardation. (*Am J Obstet Gynecol* 1993;168:214-27.)

Key words: Low-dose aspirin, preeclampsia, intrauterine growth retardation

In most countries hypertensive disease during pregnancy appears to be the largest single cause of maternal death.¹⁻³ The impact of maternal hypertension on the fetus remains disputed because of differences in criteria of selection and diagnosis between various studies. According to the World Health Organization, hypertensive disease during pregnancy is a major cause of perinatal mortality and morbidity.^{2,4} Indeed, there is no doubt that the presence of hypertension plus proteinuria is accompanied by a perinatal mortality that is substantially higher than that in normotensive pregnancies. However, hypertension without proteinuria has been shown to carry a perinatal mortality that is similar to or perhaps even lower than that in normotensive pregnancies.² Thus prevention of preeclampsia would have great implications regarding both maternal and perinatal outcomes. The terminology used to describe the pregnancy-induced form of hypertension has been

confusing and inconsistent. For the sake of clarity, this review will adhere to the definitions used in the majority of reviewed articles. The diagnosis of pregnancy-induced hypertension requires the presence of increased blood pressure criteria on two occasions at least 6 hours apart. The increase in blood pressure could be either an absolute value of $\geq 140/90$ mm Hg or a relative value, whereby blood pressure must increase >30 mm Hg systolic or ≥ 15 mm Hg diastolic from a previous recording before 20 weeks' gestation. Severe pregnancy-induced hypertension requires a diastolic blood pressure ≥ 110 mm Hg. Preeclampsia requires the presence of pregnancy-induced hypertension with significant proteinuria.

Secondary prevention of a disease requires knowledge of pathophysiologic mechanisms, availability of methods of early detection, and means of intervention and correction of pathophysiologic changes. Methods for early detection of preeclampsia were recently reviewed in some detail.⁵ The current review concerns means of pharmacologic intervention and correction of the pathophysiologic changes that occur in preeclampsia. The first section of this review presents a brief survey of some developments in our understanding of

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the pathogenesis of this enigmatic disease. The second part reviews the effects of low-dose aspirin in pregnant women and its potential value in preventing preeclampsia and fetal growth retardation. In addition, the basic pharmacologic aspects of aspirin with regard to its effects on platelet-vessel wall interaction and its possible adverse effects on the mother, fetus, and neonate will be summarized.

Preeclampsia is associated with vasospasm, pathologic vascular lesions within multiple organ systems including the uteroplacental vascular bed, and increased platelet activation with platelet consumption and subsequent activation of the coagulation system in the microvasculature.⁶ Endothelial cell injury and altered endothelial cell function play an important role in the pathogenesis of preeclampsia.⁷⁻¹¹ Indeed, evidence of endothelial injury is provided by the characteristic morphologic lesions of preeclampsia: glomerular endotheliosis¹² and ultrastructural changes in placental bed and uterine boundary vessels.¹³

The etiology of preeclampsia remains unknown, but in recent years evidence has been adduced to support the hypothesis that the eicosanoid system plays an important part in the pathophysiologic mechanisms involved in the development of its various signs and symptoms.¹⁴⁻¹⁶ Results of biochemical studies suggest that a pathophysiologic functional imbalance between vasodilator and vasoconstrictor eicosanoid products could be of major importance in this respect.¹⁷ With regard to preeclampsia, prostacyclin (PGI_2) and thromboxane A_2 (TXA_2) are the most relevant eicosanoids. TXA_2 , the principal cyclooxygenase product of arachidonic acid in platelets, is a potent vasoconstrictor and a stimulant to platelet aggregation. On the other hand, PGI_2 , the principal cyclooxygenase product of the vascular endothelium, has opposite effects on platelet function and vascular tone. Fitzgerald et al.¹⁸ reported that a reduction in the urinary excretion of prostacyclin metabolites precedes the development of clinical disease. This reduction in the urinary excretion of prostacyclin metabolites was already detectable in the first trimester. They also reported that TXA_2 biosynthesis was increased in preeclampsia, as demonstrated by significantly higher urinary excretion of TXB_2 metabolites. In addition, they demonstrated that urinary excretion of TXB_2 metabolites correlated with mean arterial blood pressure, plasma lactate dehydrogenase, and platelet count, which were used as indicators of the severity of preeclampsia.¹⁹

The imbalance between vasodilator prostaglandins and vasoconstrictors such as TXA_2 and angiotensin II, especially in the uteroplacental circulation and the kidney, may be pivotal in the development of preeclampsia. The absence of the normal stimulation of the renin-angiotensin system, despite significant hypo-

volemia, and the increased vascular sensitivity to angiotensin II and norepinephrine can be explained by a single mechanism: endothelial cell injury causing a deficiency in production and/or activity of vasodilator prostaglandins, in particular, PGI_2 . In addition, the increased TXA_2 -to- PGI_2 ratio, observed by various investigators in many maternal and fetal tissues, may be the cause of the selective platelet destruction, sometimes accompanied by microangiopathic hemolysis, and the reduced uteroplacental blood flow, arterial thrombosis, and placental infarction.¹⁶

Why is PGI_2 synthesis decreased in preeclampsia? Plasma concentrations of free-radical oxidation products were found to be significantly elevated in preeclampsia.²⁰ Wickens et al.²⁰ found a correlation between rising blood pressure and increased free radical activity. Because of the well-known effects of free radical oxidation products on vascular PGI_2 synthesis, platelet aggregation, and clotting,²¹ it is possible that in the pathogenesis of preeclampsia free radical activity contributes to the PGI_2 deficiency. In a recent study, Dekker and Kraayenbrink²² investigated lipid peroxides, as a measure of overall oxidative stress, in pregnant women with and without preeclampsia. Lipid peroxide levels increased slightly during normotensive pregnancy; however, in preeclampsia, lipid peroxide levels were nearly twice the level found in normotensive pregnant women. Longitudinal studies are currently under way to assess whether oxygen free radicals are involved in the early pathogenesis of preeclampsia.

It should be noted that, although the concept of a PGI_2 / TXA_2 imbalance allows an explanation for many of the clinical features of preeclampsia, it remains unproved if the disturbed PGI_2 / TXA_2 balance is the only pathogenetic mechanism involved. In addition, it is unknown if PGI_2 is the major physiologic vasodilator in normotensive pregnancy. Recent reports suggest that most of the eicosanoids, especially PGI_2 and prostaglandin E_2 , are operative in situations where tissue perfusion is endangered (shock, sepsis, hypoxia, etc.); in other words synthesis of these vasodilator eicosanoids can be considered a rescue mechanism.^{23, 24} It might be that the physiologic vasodilation of pregnancy is mainly or at least partially mediated by other autacoids. Endothelium-derived relaxing factor (EDRF) has recently been shown to be a major vasodilator in man. In contrast to the effects of prostaglandin synthesis inhibitors, suppression of the endothelial release of EDRF in man increases systemic blood pressure.²⁵ In hypertensive (nonpregnant) blood vessels, the ability to release EDRF but not endothelium-derived contracting factors (superoxide anions, endothelin) is blunted.²⁴ In addition, the physiologic decrease in blood pressure in pregnant, spontaneously hypertensive rats has recently been shown to depend completely on endothelial EDRF

release.²⁵ Moreover, Myatt et al.²⁶ demonstrated that the human fetal-placental circulation has the capacity to generate nitric oxide intracellularly and that nitric oxide acts as a vasodilator in this circulation. Furthermore, Pinto et al.²⁷ reported that the endothelial release of EDRF by human umbilical vessels was impaired in preeclampsia as compared with normotensive pregnancy.

Whether the endothelial cell injury in preeclampsia primarily causes a decrease in PGI₂ synthesis or a decrease in EDRF (for example), platelet dysfunction seems to play a central role in the disease process. In a recent review, Redman²⁸ stated that "preeclampsia is a trophoblast-dependent process mediated by platelet dysfunction." In the absence of an adequate production of antiaggregatory PGI₂ and/or EDRF by the uteroplacental vasculature and/or endovascular trophoblast, surface-mediated platelet activation may be expected to occur.²⁹ Platelets will adhere and release α -phase and dense granule constituents. As a result, TXA₂ and serotonin will be generated and more circulating platelets will be recruited. Coagulation will be triggered and thrombin will be locally generated, contributing to platelet aggregation and inducing the formation of fibrin to stabilize the platelet thrombus that may occlude maternal blood flow to the placental cotyledon, thus leading to placental infarction.

In summary, preeclampsia is characterized by a generalized disturbance in endothelial physiologic characteristics. It is currently uncertain if a decrease in either PGI₂ or EDRF biosynthesis or both is involved in its pathogenesis. However, increased platelet aggregation plays a central role in the disease process. TXA₂ biosynthesis is increased in preeclampsia; this increase largely derives from platelets and correlates with the severity of the disease process.

Acetylsalicylic acid (aspirin) as antiplatelet agent

Synthetic salicylates are a group of nonnarcotic analgesics that were introduced into clinical practice at the end of the nineteenth century. Since then they have been used extensively as prescription drugs and in the form of "over-the-counter" preparations to treat headaches, fever, and various aches and pains. Acetylsalicylic acid (aspirin) is by far the most commonly used drug in this group, either alone or in combination with other drugs such as acetaminophen, caffeine, or phenacetin. Headaches and minor aches and pains are common complaints during pregnancy. Hence it is not surprising that salicylates have been widely used during pregnancy. Aspirin is the most commonly used medicinal agent in the western world, and more than 20 to 30 billion tablets are consumed annually in the United States alone.³⁰ The therapeutic efficiency of aspirin as an analgesic, antipyretic, and antiinflammatory drug has been universally established for many decades be-

fore Vane³¹ discovered that aspirin acts by prevention or reduction of prostaglandin biosynthesis through inhibition of a key enzyme, cyclooxygenase.

Several epidemiologic studies of prenatal drug ingestion indicate that aspirin is the most common drug taken during pregnancy. The drug is often self-administered because of its availability as "over-the-counter" medication. In a survey of 311 randomly selected women in Scotland, 54% admitted using salicylates during pregnancy.³² In a cohort study of 50,282 pregnant women in the Collaborative Perinatal Project, 30% of the women reported using salicylates in the first trimester, and 64% reported using them at some time during pregnancy.³³ In a recent longitudinal prospective study of 1523 pregnant women receiving prenatal care in Seattle, 46% reported taking aspirin during the first half of pregnancy.³⁴

Aspirin inhibits platelet adhesion to collagen under conditions of stasis or low flow. It also inhibits "irreversible" or second-wave aggregation and the associated release reaction induced by agents such as collagen, adenosine diphosphate, and epinephrine.³⁵ Platelets obtained from subjects taking single doses of aspirin demonstrate impaired aggregation response to epinephrine, adenosine diphosphate, and collagen. The platelet aggregation response to adenosine diphosphate and epinephrine after aspirin intake is limited to a single wave of "reversible" aggregation, and both the "oxygen burst" and the release of serotonin, adenosine diphosphate, adenosine triphosphate, platelet factor 4, and TXA₂ is abolished.^{30, 35, 36} The effects of aspirin on thrombin-induced aggregation and secretion are dose-related; inhibition occurs at low but not at high thrombin concentration.³⁷

Aspirin prolongs the bleeding time through its inhibition of platelet cyclooxygenase activity and the resultant platelet secretory reaction.³⁸ It acetylates the alanine residue at the active site of platelet cyclooxygenase.^{35, 36} Consequently, the inhibition of the enzyme caused by aspirin is irreversible since platelets lack nuclei and are unable to resynthesize cyclooxygenase. Therefore, after aspirin administration, TXA₂ synthesis in platelets remains impaired for the duration of their life span.

Because the acetyl moiety is absent, salicylic acid does not inhibit the cyclooxygenase pathway *in vitro* and has no measurable effect on platelet aggregation at concentrations achieved *in vivo*.^{30, 39} However, salicylate has a potent antiinflammatory effect and is as active as aspirin *in vitro* in decreasing prostaglandin synthesis. Hence, salicylates may inhibit prostaglandin synthesis by other pathways. Salicylate also partially prevents the inhibitory action of aspirin, especially on vascular cyclooxygenase activity. After pretreatment with salicylate, platelet cyclooxygenase is significantly more sensitive to the inhibitory action of aspirin than vessel wall cycloo-

xygenase.⁴⁰ Thus salicylate seems to preferentially protect the vessel wall from the assault of aspirin. As a result, progressive accumulation of salicylate during chronic treatment with aspirin might result in a condition in which the synthesis of TXA₂ is prevented much more than that of PGI₂.

Since the precise role of PGI₂ and TXA₂ in the pathogenesis of thromboembolic diseases is still unknown, it is important to reconsider whether the clinical benefits of aspirin may be due in part to its other, previously recognized antithrombotic properties. Adenosine diphosphate is a proaggregatory substance and platelets release adenosine diphosphate by both prostaglandin-dependent and prostaglandin-independent pathways. Human vascular tissue contains an endothelial cell adenosine phosphatase, which catabolizes adenosine diphosphate to adenosine, an inhibitor of platelet aggregation. Aspirin has been shown in some, but not all, *in vitro* experimental models to enhance the metabolism of adenosine diphosphate to adenosine.^{41, 42} It may therefore act as an antithrombotic agent first by inhibiting platelet prostaglandin-dependent adenosine diphosphate release and second by increasing conversion of platelet proaggregatory adenosine diphosphate, released by platelets of prostaglandin-independent pathways, to antiaggregatory adenosine.

Recently, Buchanan et al.⁴³ showed that salicylate inhibits 12-hydroxyeicosatetraenoic acid production in the platelet, by inhibiting the cytosol-associated peroxidase that normally converts 12-hydroxypentaecosa-tetraenoic acid to 12-hydroxyeicosatetraenoic acid. Probably impairment of 12-hydroxyeicosatetraenoic acid production decreases platelet adhesion. Aspirin may also indirectly suppress the synthesis of lipoxygenase products by enhancing the effects of endogenous lipoxygenase inhibitors such as 15-hydroxyeicosatetraenoic acid.⁴⁴

High doses of aspirin (1 to 2 gm/day) prolong the prothrombin time after 2 to 3 days.⁴⁵ Daily administration of 100 and 300 mg of aspirin has no effect, but doses of 1 to 2 gm decreases the level of coagulation factors II, III, IX, and X. The mechanism is unclear, but vitamin K corrects this defect.⁴⁶ In addition, salicylate (900 to 2400 mg) increases the fibrinolytic activity of whole blood in a dose-dependent manner through increased leukocyte fibrinolytic activity.^{30, 47} Although we do not know the exact importance of these actions of aspirin, at present it is the inhibition of platelet cyclooxygenase that is thought to convey the major part of its antithrombotic and antiplatelet effects.

Importance of dosage of aspirin

The inhibitory effects of aspirin on platelet activity and hemostasis have led to its use as an antithrombotic agent.³⁰ It has been shown to be effective in the prevention of thrombosis of prosthetic valves and coronary

bypasses and in the prevention of myocardial infarction and other arterial or venous thrombotic lesions.^{48, 49} The optimal antithrombotic dose of aspirin remains unknown. Doses as high as 3.5 gm/day and as low as 20 to 40 mg/day have been reported to be effective in preventing thrombotic events.^{30, 48, 50} Obviously, the lowest effective dose of any drug is to be preferred, but in the case of aspirin this may be a particularly important issue because of its concomitant effect on vessel wall cyclooxygenase.⁵¹ Aspirin inhibits endothelial cyclooxygenase, but the vessel wall is probably less sensitive than the platelet and it has the capacity to generate new cyclooxygenase activity when aspirin is removed from the system. The endothelial recovery process is also dependent on protein synthesis and reflects the production of new cyclooxygenase.

Technically difficult studies in which PGI₂ and TXA₂ were measured both *in vivo* and *in vitro* have suggested that doses of aspirin in excess of 80 mg daily substantially inhibit both PGI₂ and TXA₂ synthesis and that only low doses of aspirin, in the region of 30 to 40 mg daily or on alternate days, would achieve an optimum balance.^{50, 52, 53} In fact, TXA₂ formation by the platelet can be inhibited by a small dose of aspirin. A daily dose of aspirin of 0.45 mg/kg given for 7 days produces a cumulative and virtually complete inhibition of platelet TXA₂ production without significantly reducing the urinary excretion of PGI₂ metabolites in healthy men and women.⁵⁴ On the contrary, Fitzgerald and Sherry⁵⁵ found that even a very low dose of aspirin (20 mg/day for 1 week) caused not only a mean reduction of 70% in urinary TXB₂ excretion but also a minor but significant reduction in urinary excretion of PGI₂ metabolites. These investigators concluded that no dose of aspirin was entirely selective of TXA₂ synthesis inhibition and that selectivity of TXA₂ versus PGI₂ synthesis inhibition was most evident in the range of 20 to 160 mg/day. Although most investigators found platelet cyclooxygenase to be more sensitive than endothelial cyclooxygenase to aspirin, this has not been confirmed in other studies.^{30, 55} Variations in these findings may reflect differences between test conditions and their influence on the capability of the endothelium to produce PGI₂.⁵⁰

In the current dispute on the effects of "low" versus "high" doses of aspirin, the pharmacokinetics of the drug should be taken into consideration. Aspirin is a weak acid (pK_a 3.5) and therefore exists primarily in the ionized form in blood or body tissues. It spontaneously hydrolyzes to sodium salicylate and the rate of hydrolysis is proportional to the pH.⁵⁶ The pharmacokinetics of both aspirin and salicylate are best described by a two-phase model. The half-lives of the α -phase of aspirin and salicylate are 2.7 and 3.8 minutes, respectively, and the half-lives of the β -phase are 15 and 238 minutes, respectively.⁵⁶ The slow elimination of the β -phase of salicylate reflects the high degree of plasma

protein binding. The excretion and metabolism of salicylate are rate limited by the unbound plasma concentration.

Aspirin is rapidly absorbed from the stomach and upper intestine. The rate of absorption is generally most rapid with aqueous solutions, less rapid with uncoated plain tablets, and slowest for enteric-coated and extended-release tablets.⁵⁷ Aspirin is then hydrolyzed to salicylic acid by intestinal, hepatic, and plasma esterases.⁵⁸ Recently, Pedersen and Fitzgerald⁵⁸ investigated the dose-related kinetics of aspirin in nonpregnant subjects using a deuterated aspirin analogue. They found that the fractional systemic bioavailability of aspirin was constant after single oral doses of 20, 40, 325, and 1300 mg of aspirin. Systemic bioavailability was also similar after single dose and long-term administration of 325 mg doses of aspirin. In addition, the authors reported that platelets passing through the gut capillaries were exposed to concentrations of aspirin that were significantly higher than those of the platelets in the peripheral circulation. Thus it has been suggested that the use of low doses of aspirin causes relatively high concentrations in the presystemic (portal) circulation leading to inhibition of cyclooxygenase in platelets passing through the gut capillaries, whereas the concentration of aspirin in the peripheral circulation remains too low to affect the enzyme in the vascular endothelium.⁵⁹⁻⁶¹ Once platelets have been inactivated in the portal circulation, they remain so for several days after the last oral dose of aspirin; restoration of total body platelet cyclooxygenase will then depend on the inflow of new platelets.

Safety aspects of use of aspirin in pregnancy

Aspirin is the most frequently ingested drug in pregnancy, either as a single agent or in combination with other drugs.⁶¹ In eight surveys totalling >54,000 patients, aspirin was consumed at some time during gestation by slightly more than 33,000 (61%).^{32, 61-68} The true incidence is probably much higher than this because many patients do not remember having taken aspirin or consume drug products without realizing that they contain large amounts of salicylates.^{61, 62} Evaluation of the maternal and fetal effects of aspirin is thus difficult because of this common and often hidden exposure. However, some toxic effects on the mother and fetus from large doses of salicylates have been known since 1893.⁶⁹

There are numerous reports describing an association between prenatal aspirin ingestion and several adverse effects in the mother, fetus, and newborn. The majority of these studies were epidemiologic in nature, most were retrospective, and all of them included the intake of regular or high doses of aspirin.^{61, 62, 64, 70-77} The reported maternal side effects have included anemia, antepartum and postpartum hemorrhage, pro-

longed bleeding time, and prolonged gestation and labor. Aspirin is readily transferred from mother to fetus across the placental barrier, and significant salicylate concentrations can be found in the cord blood and neonate.⁶² Reported fetal effects from the use of aspirin in pregnancy have included congenital defects (mainly cardiac), oligohydramnios, fetal growth retardation, and increased stillbirths. To date, only one case of in utero closure of the ductus arteriosus has been reported in association with the use of high doses of aspirin in pregnancy.⁷⁷ Neonatal complications have included various neonatal bleeding disorders, intraventricular hemorrhage in preterm infants, and persistent pulmonary hypertension.^{73, 78, 79} In contrast, the Collaborative Perinatal Project prospectively monitored 50,282 mother-child pairs, 64% of whom used aspirin at some time during pregnancy.³³ The findings of this study revealed no differences in number of stillbirths, number of neonatal deaths, or neonatal birth weights between those exposed and those not exposed to aspirin.

Several studies have examined the possible association between maternal use of aspirin and congenital defects with findings either supporting or refuting such a relationship.⁸⁰ In a retrospective survey of 599 children with oral clefts, the use of salicylates in the first trimester was found to be three times more frequent in mothers of children having this defect.⁸¹ In another survey of 298 mothers of children with congenital heart disease and 738 mothers of normal children, Zierler and Rothman⁸² suggested that first-trimester use of aspirin may increase the risk of various congenital cardiac anomalies, particularly aortic stenosis, coarctation of the aorta, and hypoplastic left heart syndrome. In addition, Agapitos et al.⁸³ reported an association between fetal cyclopia and daily maternal ingestion of up to 4 gm of aspirin in the first trimester. In contrast, the data from the Collaborative Perinatal Project did not demonstrate an increased incidence of congenital fetal defects among 14,864 women exposed to aspirin in the first trimester.⁶⁵ Moreover, Werler et al.,⁸⁴ using data from a large program of case-control surveillance of congenital malformations, concluded that aspirin use during the first trimester of pregnancy does not increase the risk of congenital heart defects.

Two studies investigated the IQ of children exposed to aspirin in utero during the first half of pregnancy.^{34, 85} In one study³⁴ a significant and negative association was found between aspirin exposure and child's IQ and the children's attentional decrements when they were examined at 4 years of age. However, a larger study with data collected in the Collaborative Perinatal Project found higher mean IQs at age 4 years in children exposed to aspirin than in those not exposed to aspirin.⁸⁵ Thus any potential adverse effect of in utero aspirin exposure on subsequent children's IQs appears unlikely. In addition, those studies con-

cerned exposure to regular doses of aspirin during pregnancy.

Low-dose aspirin

Ylikorkala et al.⁸⁶ determined PGI₂ and TXA₂ generation by umbilical arteries, neonatal urinary excretion of 6-keto-prostaglandin F_{1α}, and fetal platelet TXA₂ synthesis after administration of a single dose of 100 or 500 mg of aspirin to healthy parturients. They found significantly higher levels of salicylates in cord blood after 500 mg dose than after the 100 mg dose. Fetal and neonatal PGI₂ levels were significantly reduced after 500 mg of aspirin, but they were unchanged in infants of mothers receiving 100 mg of aspirin. In contrast, fetal TXA₂ synthesis was reduced after both 100 and 500 mg of aspirin; the latter dose was accompanied by reduced urinary excretion of PGI₂ metabolites in the neonates during the first 3 days of life. Maternal ingestion of 100 mg of aspirin had no effect on neonatal urinary excretion of PGI₂ metabolites. The authors concluded that a small dose of maternal aspirin (100 mg) inhibits only fetoplacental TXA₂ but leaves PGI₂ production unaffected. In a randomized double-blind placebo-controlled study, Sibai et al.⁸⁷ evaluated the effects of low-dose aspirin on maternal and neonatal plasma 6-keto-prostaglandin F_{1α}, platelet aggregation, platelet TXA₂ production, and neonatal transitional circulation. Forty women, at a mean of 37 weeks' gestation, were randomized (*n* = 10 each) to receive placebo or 20, 60, or 80 mg of aspirin per day until delivery. Maternal serum 6-keto-prostaglandin F_{1α} levels were not affected by these doses of aspirin, whereas TXB₂ generated during clotting of maternal blood was decreased significantly by 60 and 80 mg of aspirin at 1 week of therapy. Maternal platelet TXB₂ production in response to adenosine diphosphate or collagen was also reduced 98% by the 80 mg dose after 1 week of aspirin therapy. The 60 mg dose reduced maternal platelet TXB₂ production in response to adenosine diphosphate (50% decrease) or collagen (60% decrease) after 1 week of treatment, with a 97% decrease after 2 weeks of therapy. Neonatal serum levels of 6-keto-prostaglandin F_{1α} and TXB₂ were not affected by any dose of aspirin. Further, neonatal platelet aggregation and TXB₂ generation in response to platelet stimulation by collagen and ADP were not impaired. There were no maternal or neonatal hemorrhagic complications reported. All neonates had echocardiographic evidence of a patent ductus arteriosus, and noninvasive estimates of pulmonary arterial pressure were similar among the groups of infants. In addition, 20 to 80 mg of aspirin per day did not affect duration of pregnancy or labor. The absence of any maternal and neonatal hemorrhagic complications, as well as the absence of neonatal cardiovascular problems, agrees with the earlier findings of the Rotterdam low-dose aspirin study⁸⁸ and was recently sup-

ported by studies reported by Schiff et al.,⁸⁹ McParland et al.,⁹⁰ and Viinikka et al.⁹¹

Walsh¹⁵ and Nelson and Walsh⁹² studied the effects of aspirin on placental eicosanoid metabolism. Production rates of PGI₂ and TXA₂ in whole villi, villous core tissues, and trophoblast from human term placentas were measured in the presence or absence of aspirin. Aspirin was shown to inhibit TXB₂ production in whole villi and villous core tissue denuded of its trophoblast cells.⁹² The same concentration of aspirin also inhibited PGI₂ production in the isolated villous core but not in whole villi or isolated trophoblast. The authors concluded that low-dose aspirin can selectively inhibit TXB₂ production in whole placental villi and differentially affects TXB₂ production by the trophoblast and villous core compartments. In addition, they suggested that the trophoblast modulates villous core TXB₂ production by a paracrine interaction, and they speculated that aspirin mimics this trophoblast-inhibiting effect on TXB₂ by the placenta. Aspirin selectively inhibits TXB₂ not only in whole placental tissue but also in placental arteries.⁹³ Low-dose aspirin was shown to cause a significant decrease in TXB₂ in whole placental tissue and also in placental arteries. In addition, low-dose aspirin caused a significant decrease in TXB₂ production in placental arteries both with and without vasoconstricting doses of angiotensin II whereas PGI₂ production in placental arteries was unaffected by the same dose of aspirin.⁹³

In summary, low-dose aspirin effectively inhibits platelet TXA₂ biosynthesis; vascular PGI₂ production remains relatively unaltered. The (relative) refractoriness of systemic vascular PGI₂ biosynthesis to the effects of low-dose aspirin is based on the cell biologic difference between endothelial cells and platelets and on deacetylation of aspirin in the portal circulation. The chronic or intermittent consumption of high doses of aspirin by pregnant women may affect maternal and newborn hemostatic mechanisms, and may be associated with increased perinatal morbidity; teratogenic effects are unlikely. It appears prudent not to encourage the use of analgesic doses of aspirin in pregnancy for trivial reasons. However, the reported adverse effects are relatively rare and dose related; there is no evidence that low-dose aspirin carries any significant maternal or fetal risks. Nevertheless, if treatment of pregnant women with low-dose aspirin is considered, it should be started after 12 weeks' gestation, when the risks of inducing congenital heart disease are minimal.

Low-dose aspirin: Effect on angiotensin II sensitivity and prostaglandins

It is well established that normal pregnancy is characterized by increased refractoriness to infused vasoactive substances such as angiotensin II, whereas preeclampsia is characterized by increased sensitivity to

Table 1. Low-dose aspirin and vascular angiotensin II sensitivity in pregnancy

Series	No. of women	Gestational age at initial infusion (wk)	EPD before aspirin (ng/kg/min)	EPD after aspirin (ng/kg/min)	Duration of aspirin treatment
<i>Angiotensin II nonsensitive women</i>					
Sanchez-Ramos et al. ⁹⁶	13*	28-34	17.2 ± 2.2	35.1 ± 4.2	2 hr
Brown et al. ⁹⁸	18	28-32	25.5 ± 10.6	25.5 ± 10.6	1 wk
<i>Angiotensin II-sensitive women</i>					
Spitz et al. ⁹⁷	17*	32.7 ± 2.3	5.9 ± 2.4	10.2 ± 5.5	1 wk
Brown et al. ⁹⁸	22	28-32			
SN	11*	31.3 ± 2.6	6.3 ± 2.5	18.1 ± 7.9	1 wk
SS	11	32.5 ± 1.6	5.0 ± 2.5	4.7 ± 1.9	1 wk
Wallenburg et al. ⁹⁹	32	28	7.5	—	—
Aspirin	17	28	7.5	11†	6 wk
Placebo	15	28	7.5	7.5†	6 wk

EPD, Effective pressor dose of angiotensin II. Results are mean ± SD except for Wallenburg et al. (median). SN, Women sensitive before and nonsensitive after low-dose aspirin. SS, Women sensitive before and after low-dose aspirin.

* $p < 0.01$ for effective pressor dose before and after aspirin.

† $p < 0.02$.

angiotensin II infusions.⁵ In addition, Gant et al.⁹⁴ demonstrated that women destined to have preeclampsia lose their refractoriness to infused angiotensin II several weeks before the onset of the clinical manifestations of the disease. Moreover, Everett et al.⁹⁵ found that the vascular response to angiotensin II infusions in normotensive women is altered after the use of regular doses of aspirin.

There are several studies describing the effects of low-dose aspirin (60 to 81 mg/day) on angiotensin II sensitivity during pregnancy. Three of these studies investigated the effects of aspirin in pregnant women who were either sensitive or nonsensitive (refractory) to angiotensin II infusions,⁹⁶⁻⁹⁸ and one study investigated the effects of aspirin and placebo in sensitive pregnant women⁹⁹ (Table 1).

Spitz et al.⁹⁷ evaluated the effects of low-dose aspirin (81 mg/day for 1 week) on angiotensin II pressor responses and blood prostaglandin concentrations in 17 women sensitive to angiotensin II. Low-dose aspirin resulted in a substantial increase in the effective pressor dose of angiotensin II; however, the observed values remained lower than those recorded in a nonsensitive group of women. In addition, the authors noted that in spite of the increase in the effector dose of angiotensin II after aspirin therapy 8 of 17 women (47%) remained sensitive (<10 ng/kg per minute) to angiotensin II. Moreover, they found that the use of low-dose aspirin resulted in significant reductions in the levels of serum and plasma TXB₂, plasma 6-keto-prostaglandin F_{1α}, and plasma prostaglandin E₂. However, the decreases in plasma TXB₂ were significantly greater than the decreases in plasma 6-keto-prostaglandin F_{1α}, resulting in an increased 6-keto-prostaglandin F_{1α}/TXB₂ ratio. They concluded that low-dose aspirin affects platelet, vascular, and uteroplacental production.

Brown et al.⁹⁸ studied pregnancy outcomes in 40 pregnant women (28 to 32 weeks' gestation) who were given low-dose aspirin (81 mg/day) from the time of

enrollment until delivery. Circulating eicosanoid levels and angiotensin II pressor responses in these women were measured before and after 1 week of aspirin therapy. Eighteen women were classified initially as nonsensitive to angiotensin II whereas 22 women were sensitive to angiotensin II infusion at the time of enrollment. All studied women had significant reductions in serum and plasma TXB₂ levels after therapy. Eleven of the 22 women initially sensitive to angiotensin II remained sensitive after 1 week of aspirin therapy; these women had significant reductions in both plasma 6-keto-prostaglandin F_{1α} and prostaglandin E₂ levels. In contrast, such reductions in these prostaglandin metabolites were not demonstrated in the women who were initially nonsensitive to angiotensin II or in the 11 women who became nonsensitive to angiotensin II infusions after aspirin therapy. In all 11 women who remained sensitive to angiotensin II pregnancy-induced hypertension developed: Four had mild disease, five had severe disease, and two (18%) had preeclampsia. In addition, these 11 women required delivery at a mean gestational age of 35.6 weeks. On the other hand, in 4 of the 11 women who became nonsensitive after aspirin therapy pregnancy-induced hypertension developed: One had mild disease, one had severe disease, and two (18%) had preeclampsia. Interestingly, the incidence of pregnancy-induced hypertension (39%) was significantly high in the 18 women who were nonsensitive to angiotensin II at onset of the study: Four had mild disease, one had severe disease, and two (11%) had preeclampsia. In addition, five women within this group became more sensitive to angiotensin II after aspirin therapy; pregnancy-induced hypertension developed in four of the five. The authors suggested that pregnant women may have different biochemical responses to the effects of low-dose aspirin therapy; some women may have nonselective inhibition of eicosanoids resulting in adverse pregnancy outcome.

Wallenburg et al.⁹⁹ conducted a double-blind placebo-

bo-controlled study using low-dose aspirin (60 mg/day for 6 weeks; $n = 17$) or a placebo ($n = 15$) in normotensive primigravid women who were sensitive to angiotensin II infusions at 28 weeks' gestation. All women had measurements of thrombin-induced platelet malondialdehyde production and repeat angiotensin II infusions at 34 weeks' gestation. In the aspirin-treated group the values of platelet malondialdehyde production were significantly lower than in the placebo group. In addition, the effective pressor dose of angiotensin II at 34 weeks' gestation was significantly higher than at 28 weeks in the aspirin group but not in the placebo group. Moreover, seven women in the placebo group and none in the aspirin group had a decrease in the effective pressor dose between 28 and 34 weeks' gestation. Further, vascular refractoriness to angiotensin II (effective pressor dose of > 10 ng/kg/minute) was restored in 14 of 17 aspirin-treated women but in only 5 of 15 placebo-treated women.

In summary, the data regarding the effects of low-dose aspirin on angiotensin II vascular sensitivity during pregnancy are highly variable. However, the data suggest that enhanced vascular responsiveness to angiotensin II infusions may be mediated by an imbalance in thromboxane/PGI₂ production that may be corrected by the use of low-dose aspirin.

Prevention of preeclampsia and growth retardation

With the development of the pathophysiologic concept of selective, non-thrombin-mediated platelet activation in women with preeclampsia, antiplatelet drugs have been considered for both prevention and treatment. Preeclampsia is just one expression of the circulatory maladaptation disease of pregnancy. Fetal growth retardation and abruptio placentae may be the other manifestations, occurring alone or in combination with preeclampsia. Normotensive pregnant women with fetal growth retardation show signs of inadequate plasma volume expansion. In addition, vascular sensitivity to angiotensin II is increased in normotensive pregnancies complicated by fetal growth retardation.¹⁶ Moreover, uteroplacental vascular changes are known to be the same in preeclampsia and fetal growth retardation if caused by uteroplacental insufficiency.¹⁰⁰ Therefore the use of low-dose aspirin has also been considered for prevention and/or treatment of fetal growth retardation.

Prevention of preeclampsia

Crandon and Isherwood¹⁰¹ obtained a history of aspirin intake during pregnancy from primigravid women and showed that in women who had taken aspirin more than once every 2 weeks through pregnancy, pregnancy-induced hypertension was significantly less common (4%) than in women who had no history of aspirin ingestion (16%). However, those authors did not subdi-

vide the aspirin-taking group by dosage or gestational age at which they began to take aspirin, nor did they present data showing that the control group was comparable to the experimental group in terms of gravidity, blood pressure at initial visit, or incidence of coexisting medical conditions.

Several prospective studies have recently been published which suggest that aspirin administration might reduce the incidence of preeclampsia. The first prospective randomized trial was an open study in which patients who had a history of a complicated pregnancy and/or blood pressure $\geq 160/95$ mm Hg were randomized to receive either aspirin (150 mg) and dipyridamole (300 mg) daily, starting at 3 months' gestation, or no treatment.¹⁰² Results of this trial are summarized in Table II. No fetal loss occurred in the women treated with aspirin and dipyridamole, whereas five fetal deaths occurred in the control group. Placental weights showed no significant difference between the treated and the untreated groups. The authors concluded that antiplatelet therapy begun early in pregnancy in high-risk patients significantly improves maternal and fetal outcome. However, this study had several methodologic problems: (1) Its applicability to "true preeclampsia" was questionable because the vast majority of the subjects were multiparous; (2) the rate of pregnancy complications, including preeclampsia, seen in the control group was unexpectedly high; (3) the number of patients with preexisting hypertension was higher in the control group than in the study group (38 vs 29%); (4) the authors did not report on the levels of proteinuria during the first trimester in those patients subsequently labeled as having preeclampsia; (5) the study was not done in a double-blind placebo-controlled format; (6) this trial tested the combination of aspirin and dipyridamole rather than aspirin alone.

The first randomized placebo-controlled double-blind study on the possible prevention of preeclampsia in primigravid women with low-dose aspirin was reported in 1986.⁸⁸ Sensitivity to intravenously infused angiotensin II was determined at 28 weeks' gestation in 207 normotensive primigravid pregnant women. All 46 women with an effective pressor dose of ≤ 10 ng/kg/minute were enrolled in the study. At 33 to 35 weeks a venous blood sample was drawn for determination of thrombin-induced production of malondialdehyde by platelets. In the aspirin group determination of thrombin-induced production of malondialdehyde by platelets indicated that they had complied with the prescribed regimen, at least at the time that the determination was done (33 to 35 weeks' gestation), except for two women who were subsequently excluded from the analysis. The course and outcome of pregnancy in 44 of 46 women in the study group are summarized in Table II. There were more premature deliveries and small-for-gestational-age babies in the placebo group than in the aspirin group, but these differences did not

Table II. Randomized trials of low-dose aspirin to prevent preeclampsia

Series	Risk factors used	Dose of aspirin (mg)	Gestational age at enrollment (wk)	No. of patients		Pregnancy-induced hypertension				Preeclampsia			
						Placebo		Aspirin		Placebo		Aspirin	
				Placebo	Aspirin	No.	%	No.	%	No.	%	No.	%
Beaufils et al. ¹⁰²	Obstetric history	150*	12	45†	48	22	49	19	40	6	13	0	
Wallenburg et al. ⁸⁸	Sensitive to angiotensin II	60	28	23	21	4	17	2	9.5	8	35	0	
Schiff et al. ⁸⁹	Positive roll-over test result	100	28-29	31	34	4	13	3	9	7	23	1	3
Benigni et al. ¹⁰³	Obstetric history	60	12	16	17	3	19	0		0		0	
McParland et al. ⁹⁰	Abnormal uterine Doppler results	75	24	52	48	13	25	6	13	10	19	1	2
Uzan et al. ¹⁰⁴	Obstetric history	150*	15-18	73	156	25	34	35	22	8	11	5	3
Azar and Turpin ¹⁰⁵	Obstetric history	100*	16	45**	46	7	16	4	9	4	9	1	2
All studies				285	370	78	27.4	65	17.6	43	15.1	8	2.2

NA, Not available.

*Patients received aspirin plus dipyridamole.

†Control patients received no treatment instead of placebo treatment.

reach statistical significance. Blood loss during delivery was similar in both groups, and excessive bleeding did not occur in either group. Also, hemorrhagic complications and premature closure of the ductus arteriosus were not noted in the newborns.

Benigni et al.¹⁰³ conducted a randomized study in 33 women who were considered to be at risk for preeclampsia because of their obstetric histories. The aspirin-treated women had a lower incidence of pregnancy-induced hypertension, longer gestations, and higher neonatal birth weights (Table II). In addition, the study showed a partial (63%) reduction of neonatal serum TXB₂ in the infants of patients treated with aspirin. However, such an inhibition of platelet TXA₂ synthesis is generally not considered to cause a significant impairment of platelet function. To verify that the favorable effect of low-dose aspirin was associated with a selective reduction of platelet-derived TXA₂, the authors measured the urinary excretion of TXB₂ and its major urinary metabolite, 2,3-dinor-TXB₂, by mass spectrometric analysis. The urinary excretion of 2,3-dinor-TXB₂ was reduced in the patients treated with aspirin by 81% ± 12% as compared with the pretreatment values, whereas the urinary excretion of TXB₂ was reduced by 59% ± 23% as compared with pretreatment values. Aspirin did not modify the urinary excretion of 2,3-dinor-6-keto-prostaglandin F_{1α} and 6-keto-prostaglandin F_{1α} (an index of endogenous PGI₂ synthesis). The authors suggested that the favorable effects of low-dose aspirin were caused by a selective suppression of platelet TXA₂ synthesis while vascular PGI₂ synthesis remained intact.

Schiff et al.⁸⁹ studied 791 pregnant women with various risk factors for development of hypertensive

disease during pregnancy; 65 women were selected because of an abnormal pressor response during the roll-over test at 28 to 29 weeks' gestation. The number of women in whom pregnancy-induced hypertension developed was significantly lower among the aspirin-treated than among the placebo-treated women (Table II). No serious maternal and/or neonatal side effects of aspirin treatment were observed. The mean ratio of serum levels of TXA₂ to serum levels of PGI₂ metabolites after 3 weeks of treatment decreased by 34.7% in the aspirin-treated group but increased by 51.2% in the placebo-treated group. In a recent study by McParland et al.⁹⁰ women at a high-risk for development of preeclampsia were selected by means of Doppler ultrasonography. Patients with abnormal results of Doppler studies were enrolled in the study at 24 weeks' gestation (Table II). Hypertension occurring before 37 weeks' gestation developed in 17% of the placebo group but in none of the aspirin group. There were no differences between the two groups regarding gestational age at delivery, neonatal birth weights, or incidences of low-birth-weight babies (<2500 gm). There were three perinatal deaths in the placebo group that were related to severe hypertension and one perinatal death in the aspirin group, which was due to a cord accident during labor. In addition, there were no differences in mean blood loss recorded at time of delivery, and one of the infants had hemorrhagic complications.

Uzan et al.¹⁰⁴ reported on a multicenter, randomized, placebo-controlled, double-blind trial in 229 women selected on the basis of fetal growth retardation and/or fetal death or abruptio placentae in at least one previous pregnancy. The women were enrolled at 15 to 18 weeks' gestation and were allocated to receive either a

Intrauterine growth retardation			
Placebo		Aspirin	
No.	%	No.	%
13	29	4	8
9	39	4	19
6	19	2	6
6	38	2	12
7	14	7	14
19	26	20	13
NA	—	NA	—
60	25	39	12

placebo ($n = 73$), aspirin 150 mg/day ($n = 81$), or aspirin 150 mg/day plus dipyridamole 225 mg/day ($n = 75$). Pregnancy outcome was compared between the placebo and the two treatment groups combined (Table II). The mean birth weight was significantly higher in the treated group (difference of 225 gm). The difference in birth weight in the treated group was more pronounced in the subgroup with two or more previous outcomes (difference of 346 gm). In addition, the treated group had a lower incidence of perinatal deaths (8.2% vs 4.5%). One maternal death was attributed to amniotic fluid embolism in the aspirin-treated group. Epistaxis and other minor bleeding occurred in 8 women receiving aspirin but not in the group that received placebo. Neonatal bleeding episodes were infrequent in both groups.

Azar and Turpin¹⁰⁵ studied pregnancy outcome in 91 pregnant women considered at risk for preeclampsia because of previous poor pregnancy outcomes. Forty-six received aspirin 100 mg/day plus dipyridamole 300 mg/day starting at 16 weeks' gestation until delivery; the other 45 received no treatment (Table II). The mean duration of pregnancy and mean birth weights were significantly higher in the treatment group.

In summary, the data in Table II indicate that low-dose aspirin is highly effective in the prevention of preeclampsia and fetal growth retardation in women considered at risk for these complications.

Aspirin in established preeclampsia

Although low-dose aspirin was effective in preventing preeclampsia, the effects of aspirin in women with established pregnancy-induced hypertension or preeclampsia appear to be less favorable. The first reports

of aspirin use to treat preeclampsia relate to the use of relatively high doses of aspirin in two women with preeclampsia.^{106, 107} In one case a preeclamptic patient with thrombocytopenia at 22 weeks' gestation was treated with heparin and aspirin 1800 mg daily. Platelet counts showed improvement, but the pregnancy was complicated by oligohydramnios and the delivery of a growth-retarded infant at 34 weeks' gestation.¹⁰⁶ In the other case, a patient had eclampsia with disseminated intravascular coagulation at 21 weeks and was treated with aspirin 500 mg three times a day. Platelet counts improved; however, the pregnancy ended in fetal death 1 week later.¹⁰⁷ In a later study, 11 women with thrombocytopenia and preeclampsia (type B edema-proteinuria-hypertension gestosis) received 85 mg of aspirin per day in divided doses; platelet counts improved in every case, although no improvement of fetal condition was noted and there were two perinatal deaths.¹⁰⁸

Schiff et al.¹⁰⁹ conducted a randomized, double-blind trial in 47 women with mild pregnancy-induced hypertension at 30 to 36 weeks' gestation who were treated by a daily dose of either 100 mg of aspirin ($n = 23$) or placebo ($n = 24$). The mean blood pressure values, rates of development of severe preeclampsia, gestational age at delivery, and newborn weights were similar in the aspirin-treated and placebo-treated groups. The authors concluded that "low-dose aspirin was not curative but was essentially a preventive treatment which, in order to be effective, should be started weeks before clinical signs of preeclampsia develop." Heyborne et al.¹¹⁰ described three patients with HELLP syndrome (hemolysis, elevated liver enzymes, and low platelets) in whom pregnancy could be prolonged successfully with low-dose aspirin. In these patients platelet count increased and liver enzyme levels decreased after initiation of low-dose aspirin treatment. However, it is uncertain whether these beneficial effects were caused by aspirin treatment because the patients received betamethasone as additional treatment. In addition, aspirin causes a long-lasting platelet dysfunction. Thus the combination of thrombocytopenia and iatrogenic thrombocytopathy in a patient with HELLP syndrome may cause severe hemorrhagic diathesis. In fact, we have encountered several cases of fatal and near-fatal bleeding caused by attempts to treat women with HELLP syndrome with low-dose aspirin.

Prevention of growth retardation

In a controlled, nonrandomized trial reported by Wallenburg and Rotmans,¹¹¹ a treatment group of 24 multigravid women with a history of at least two previous pregnancies, all complicated by fetal growth retardation and placental infarction, received aspirin 1 to 1.6 mg/kg and 225 mg of dipyridamole daily from 16 to 34 weeks' gestation in a total of 30 pregnancies. Results

obtained in the treatment group were compared with those in 27 pregnancies among a control group of 24 multigravid women with a similar history. The control group received comparable antenatal care without any medication. Fetal growth retardation occurred in 61% of the control pregnancies and in only 13% of the treated pregnancies; severe fetal growth retardation was not observed in treated pregnancies, but it occurred in 27% of the control group. Treatment did not produce any adverse maternal, fetal, and/or neonatal effects. In this study aspirin 1 to 1.6 mg/kg per day combined with dipyridamole 225 mg/day markedly suppressed platelet TXA₂ synthesis, as indicated by a fall in thrombin-induced malondialdehyde production to 5% to 10% of the pretreatment value. The results of a similar but uncontrolled study were reported by Elder et al.¹¹² In this study the effects of low-dose (75 mg) aspirin were assessed in 42 patients with very poor obstetric histories (10% live births). In these women low-dose aspirin was associated with a striking improvement in pregnancy outcome (88% live births), especially in patients with systemic lupus erythematosus.

A randomized, placebo-controlled, double-blind trial was carried out by Trudinger et al.¹¹³ to evaluate the effects of low-dose aspirin on neonatal outcome in pregnancies with a compromised fetoplacental circulation. Forty-six women referred because of concern about fetal welfare were selected because of an elevated umbilical artery Doppler flow velocity waveform systolic/diastolic ratio. Mothers with severe hypertension were excluded. A distinction was made between women with a high systolic/diastolic ratio ($n = 34$, >95th but <99.95th percentile) and women with an extremely elevated systolic/diastolic ratio ($n = 12$, >99.95th percentile). Aspirin therapy (150 mg daily) was associated with a significant ($p < 0.02$) increase in birth weight (mean difference 526 gm) and in head circumference (mean difference 1.7 cm) in those patients with a higher initial systolic/diastolic ratio. The placental weight was significantly ($p < 0.02$) greater in the aspirin group (mean 535.4 gm) than in the placebo group (399.4 gm). The significant difference in birth weight between the treated and untreated groups in this study was remarkable because aspirin therapy was started very late in the course of uteroplacental disease; patients entered the study at about 32 weeks' gestation. For the 12 women with an extreme initial systolic/diastolic ratio (absent diastolic flow) aspirin therapy did not result in a significantly different pregnancy outcome. This absence of benefit was considered to indicate that irreversible vascular sclerosis had already occurred in the placentas of these women. In a subsequent report Trudinger et al.¹¹⁴ studied a group of 27 women with twin pregnancies, in which there was no evidence of placental insufficiency at 28 to 30 weeks'

gestation. These women were randomly allocated to receive either aspirin 100 mg/day ($n = 15$) or placebo ($n = 12$) until delivery. There were no differences between the two groups regarding gestational age at delivery, neonatal birth weights, or placental weights. The authors concluded that low-dose aspirin does not affect fetal growth or placental size in uncomplicated twin gestations.

In summary, the available clinical data suggest that low-dose aspirin is effective in reducing the incidences of pregnancy-induced hypertension (40%), preeclampsia (85%), and fetal growth retardation (50%) in women at high risk for these complications. This benefit appears to be particularly true in patients with poor obstetric history in previous pregnancies. However, the data are limited, considering only a few hundred patients have been studied in each group.

Comment

Preeclampsia remains a major cause of perinatal and maternal morbidity and mortality. Preeclampsia is characterized by generalized endothelial cell injury. TXA₂ biosynthesis is increased in preeclampsia. This reduced platelet-derived TXA₂ biosynthesis without a major impact on systemic PGI₂ production. The (partially) selective effect of low-dose aspirin on TXA₂ biosynthesis is based on cell biologic and pharmacokinetic principles. Low-dose aspirin does influence vascular sensitivity to angiotensin II in angiotensin II-sensitive women. The decreased vascular sensitivity to angiotensin II after low-dose aspirin in angiotensin II-sensitive women supports the importance of a functional PGI₂/TXA₂ imbalance in the pathophysiologic characteristics of preeclampsia. Low-dose aspirin is not curative but is essentially a preventative treatment, which, to be effective, should be started weeks before clinical signs of preeclampsia are present. Administration of low-dose aspirin has only a minor effect on the incidence of pregnancy-induced hypertension but a major impact on the incidence of the real disease, preeclampsia, in women with poor obstetric histories.

Currently, several large-scale clinical trials are in progress to determine more reliably any beneficial or adverse effects of low-dose aspirin in the prevention (and treatment) of preeclampsia. The National Institute of Child Health and Human Development multicenter study has just finished recruitment of >3000 nulliparous women in whom low-dose aspirin (60 mg/day) or a placebo has been used, starting between 13 and 26 weeks' gestation and continuing until delivery. The Collaborative Low Dose Aspirin Study in Pregnancy (CLASP) is another multicenter study being conducted from Oxford, England. This multinational study is recruiting both nulliparous women and women with poor obstetric histories in a randomized fashion to receive

either placebo or aspirin 60 mg/day. At completion of recruitment for this study >10,000 pregnant women will have been randomized. Recently, the National Institute of Child Health and Human Development started another trial in which pregnant women with high-risk characteristics (previous preeclampsia-eclampsia, chronic hypertension, class B to F diabetes, or multiple gestation) are being randomized to either aspirin 60 mg/day or a matching placebo. This trial will recruit >2000 pregnant women and the results will be available in early 1994. Until these studies have been completed, it will remain unclear whether antiplatelet therapy, such as low-dose aspirin, should be adopted for the prevention of either preeclampsia or fetal growth retardation. Otherwise, women receiving such therapy should have close monitoring of pregnancy for potential side effects. Monitoring of these women should include warning signs and symptoms of bleeding, serial ultrasonography for fetal growth and amniotic fluid evaluation, and neonatal evaluation for bleeding complications.

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Review

SALICYLIC ACID

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Salicylic acid is the most commonly prescribed used topical salicylate. Methyl salicylate, the active ingredient in oil of wintergreen, is present in many over-the-counter liniments used for the treatment of muscle aches and bursitis, but is usually not formulated into prescription drugs.

Pliny (first century A.D.) used willow bark (*Salix alba*) which contains salicylates to treat corns and calluses. Galen (second century) used it to treat ulcers, fistulae and erysipelas. In the early nineteenth century salicylates were not important dermatologically and did not appear in the materia medica of Willan or Hebra. In 1860, salicylates were chemically synthesized. During the decade 1880-1890 salicylates became firmly established in the dermatologic therapeutic armamentarium, and were used to soften epidermis, to act as an antipruritic, to remove freckles and even to treat lupus vulgaris. Since that era, salicylic acid has been used predominantly as an agent to remove hyperkeratotic debris.

Pharmacodynamics

The effects of salicylic acid on the epidermis will be emphasized. These effects have

been recently reviewed.¹ Standard pharmacology texts discuss the effect of salicylates on prostaglandin synthesis, metabolism and on body tissues in general.

Histological studies² of the skin during topical salicylic acid application demonstrated that desquamation is associated with intracellular and intercellular edema, intraepidermal vesicle formation and slight dermal inflammation. Intercellular gaps were visible on scanning electron micrographs of treated areas,³ and the contour of stratum corneum cells become irregular with salicylate treatment.

Salicylates denature proteins and are used to solubilize many proteins including glycoproteins;⁴ this suggests a possible mechanism for desquamation. Salicylates may solubilize cell surface proteins which keep the stratum corneum intact. The exact mechanism for desquamation is still not completely clarified.

Salicylic acid is used predominantly for its "keratolytic" effect, i.e. its ability to desquamate stratum corneum. Epidermal proliferation is also decreased.⁵ Until the mechanism of salicylic acid is more definitively defined, it is useful to retain the term keratolytic. One to three percent concentrations of salicylic acid are reported to stimulate mitoses and on this basis the drug was used as an aid in wound and ulcer healing. This was considered a "keratoplastic" effect of the drug. The drug is rarely used in this manner today.

Pharmacokinetics

Salicylic acid and methylsalicylate are both absorbed percutaneously with maximum plasma levels occurring 6-12 hours after ap-

No. 1

plication. Salicylic acid is found in extracellular space. Patients with a decreased plasma level due to e.g. dehydration have lower salicylate levels. Extracellular space is smaller on a well-hydrated person which suggests an increase in salicylate given (mg/kg) d.

Plasma levels of salicylate are absorbed orally. The percent of salicylate absorbed is 60-90 min.⁶ Salicylates compete with several drugs (T) for action of these drugs. Mechanisms of other drugs on serum level of salicylate such as may occur during exfoliative therapy with increased locally high concentration reported in muscle administration is

Salicylate is a somal enzyme s

I Drugs Changing S. Drug

Corticosteroids²⁴
Ammonium Sulfate²⁵

II Drugs with Similar Drug

Tolbutamide²⁶
Methotrexate²⁷

III Drugs with Comp. Drug

Heparin²⁸

Pyrazinamide²⁹
Uricosuric Agents^{30,31}

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plication. Salicylates are distributed in the extracellular space (150 ml/kg body weight). Patients with a contracted extracellular space due to e.g. dehydration, diuretics, have higher salicylate levels than those with a normal extracellular space. The extracellular space is smaller on a weight basis in infants and children which suggests a basis for the relative increase in salicylate levels in this group for a given (mg/kg) dose.

Plasma levels of salicylates absorbed percutaneously are additive with salicylates absorbed orally or rectally. Fifty to eighty percent of salicylate is protein bound to albumin.⁶ Salicylates compete with the binding of several drugs (Table 1) and can modify the action of these drugs; by similar competitive mechanisms other drugs can influence the serum level of salicylates. Hypoalbuminemia, such as may occur in many diseases, including exfoliative erythroderma, is associated with increased levels of free salicylates. Locally high concentrations of salicylates are reported in muscles under the site of topical administration in guinea pigs.⁷

Salicylate is metabolized by liver microsomal enzyme systems: these enzymes con-

jugate salicylic acid to glycine, form glucuronides or oxidize salicylic acid to hydroxybenzoic acids.⁸ About 65–85% of a topically administered salicylate dose is recoverable from the urine. Salicyluric acid is 52% of total recovered metabolites; the acyl and phenolic glucuronides of salicylic acid are 42% of total, and salicylic acid is the remainder.⁸ The urinary metabolites after percutaneous absorption differ from those after oral salicylate administration; those derived from percutaneous absorption contain more salicylate glucuronides and less salicyluric and salicylic acid.⁸ Almost 95% of a single dose of salicylate is excreted within 24 hours of its entrance into the extracellular space.

Clinical Pharmacology

Usage

The drug is used to remove excessively thick stratum corneum (its traditional usage), as a primary therapy or before the application of other therapeutic agents. One to two percent salicylic acid lotion is used as an antipruritic agent and there is renewed interest in

Table 1. Drug Interactions of Salicylates

I Drugs Changing Salicylate Levels by Altering Renal Tubular Reabsorption

Drug	Comments
Corticosteroids ²⁴	Decreases plasma salicylate level; with steroid taper may get salicylism
Ammonium Sulfate ²⁵	Increases plasma salicylate level, drugs acidify urine increasing salicylate tubular reabsorption

II Drugs with Similar Albumin Binding Sites as Salicylates (Salicylates Increase the level of the Drug)

Drug	Comments
Tolbutamide ²⁶	Hypoglycemia potentiated
Methotrexate ²⁷	Also ↓ tubular reabsorption, clinical toxicity of methotrexate can result

III Drugs with Complicated Interactions with Salicylates

Drug	Comments
Heparin ²⁸	Salicylates decrease platelet adhesiveness and interfere with hemostasis in heparin-treated patients
Pyrazinamide ²⁹	Decrease pyrazinamide-induced hyperuricemia
Uricosuric Agents ^{30,31}	Uricosuric effect of Probenemid, sulfapyrazone and phenylbutazone inhibited

topical salicylates as anti-inflammatory agents.⁹

It is used for wart removal as a 2–20% solution in collodion; or as a 10–40% plaster for plantar warts and plantar hyperkeratosis; in a salicylic acid soap for tinea versicolor; and as a 6% salicylic acid preparation in a 60% propylene glycol gel for hyperkeratotic diseases. Low concentrations of salicylic acid are in shampoo and other medications where they would be used to cause desquamation.

Lassar's Paste (zinc oxide paste, U.S.P. XIX) contains 25% zinc oxide, 25% starch and 50% petrolatum.

Lassar's paste as originally formulated is a paste with 2% salicylic acid, 24% zinc oxide, 24% starch; and 50% white soft paraffin is in common use as a skin protective; it has been suggested that the salicylic acid is not pharmacologically active in this medication because of its chemical reaction with zinc oxide.¹⁰ In the British Pharmacopoeia (1973) Lassar's paste is the original salicylic acid containing formula.

Whitfield's ointment with 6% salicylic acid and 12% benzoic acid in wool fat and petrolatum is occasionally used for desquamation, and as a nonspecific antifungal agent.

Gels with 8.7% choline salicylate have been used for symptomatic relief of painful aphthous ulcers.¹¹

Topical salicylates are bacteriostatic and photoprotective.¹

Clinical Application

Stripping experiments demonstrate the upper layers of the stratum corneum to be the main epidermal barrier to the passage of salicylates.¹² The rate of salicylate penetration can differ up to nine fold depending on the hydration of the skin.⁶

Vehicle composition profoundly influences the absorption of salicylic acid. The addition of polyethylene glycol 400 or polyethylene glycol 6,000 to aqueous solutions decreases the in vivo absorption of salicylic acid; a 25% (v/v) concentration of these glycols decreased absorption by 50%.¹³ The effect is probably

due to changing the stratum corneum/vehicle partition coefficient. Ethanol (50–75%) enhances penetration into guinea pig epidermis.⁷

Methyl salicylate is absorbed better from 70% alcohol/30% water solutions than from pure water solutions. Its absorption is decreased by polyethylene glycol 400 and propylene glycol.⁷

In experiments with excised human skin, salicylate penetration was best from lanolin, plastibase, and hydrophobic base, less from carbowax, and poorest from petrolatum.¹⁴

The effect of pH on absorption is also important, and the absorption of salicylic acid from hydrophilic ointment U.S.P. has been studied and extensively commented upon.¹³ Absorption was increased 50% at pH 2.98 and pH 10.7 compared with absorption at pH 4.48 and 6.8. The changes at the extremes of pH may not specifically be related to the drug, but rather to the direct effect of pH extremes on normal function of the stratum corneum, and the effect of pH on the detergent in hydrophilic ointment U.S.P.

Parameters to Monitor

Local irritation, stinging or burning may limit local usage. Since most cases of systemic toxicity have occurred with multiple applications of drug, once a day application is preferred.

As a rule of thumb, for an adult 1 g of a 6% salicylic acid preparation will raise the serum salicylate level not more than 0.5 mg/100 ml plasma. In children higher levels are possible. Calculation of the blood level by assuming 100% absorption and assuming distribution in extracellular space (150 cc/kg body weight) is an easily performed task. Instructions to patients and nurses as to the total amount of drug to be used are critical to prevent toxicity. Serum salicylate determination 6–8 hours after beginning treatment is appropriate in some patients receiving treatment to detect increased blood levels even before early signs of salicylism.

Special Pharm

Unwanted Effi

Salicylic in occurred after acid and meth tients have thir confusion, na depression and children, crying bility are early clues to diagno cases have bee degree of skin salicylate appli application, and least 11 persons ates.^{17–19} Risk f materials up to not limiting the t ates. Therefore safest especially monitored. In s which was asso rate of salicylate should also be epidermis is a p salicylates than

Symptomatic t ates is usually cases, blood ga

Test
Thyroid Function ²²
Urinary Sugar ²³
5-hydroxyindole ace
Acetone, ketone bod
17-OH Corticosteroid
Vanilmandelic Acid ²⁴
Uric Acid ²⁵
Prothrombin ²⁶
* Physiological effe

Special Pharmacological Effects

Unwanted Effects

Salicylic intoxication^{15,16} and death has occurred after percutaneous usage of salicylic acid and methylsalicylate. Clinically, the patients have thirst, tinnitus, headache, lethargy, confusion, nausea, vomiting, diaphoresis, depression and disorientation. In the affected children, crying, emotional lability and irritability are early symptoms of salicylism and clues to diagnosis. The risk factors in these cases have been the age of the patient, the degree of skin involvement, the amount of salicylate applied, the frequency of salicylate application, and the presence of occlusion. At least 11 persons have died from topical salicylates.¹⁷⁻¹⁹ Risk factors include applications of materials up to six times a day and especially not limiting the total amount of applied salicylates. Therefore, once a day application is safest especially if blood levels are not being monitored. In some cases, the use of a base which was associated with a high absorption rate of salicylate was a definite risk factor.¹⁸ It should also be remembered that diseased epidermis is a poorer barrier to the passage of salicylates than normal epidermis.

Symptomatic treatment and stopping salicylates is usually sufficient therapy. In severe cases, blood gas and electrolyte determina-

tion and fluid and electrolyte correction may be necessary for complete patient care.

Low serum levels of salicylates such as occur after topical administration can increase serum uric acid levels. This effect would be additive with thiazide diuretics and other drugs which increase serum uric acid levels. Gout may result. Significant salicylate levels can affect platelet function and alter blood coagulation. Patients allergic to salicylates have had urticarial,²⁰ anaphylactic, and erythema multiforme reactions. Pustular and acneiform eruptions have been described. Salicylates can cause morphological and chemical evidence of hepatitis in selected patients: this is reported in juvenile rheumatoid arthritis²¹ and lupus erythematosus.^{22,23}

Salicylates cross the placental barrier and in rodents are teratogenic. All possible drugs during pregnancies should be avoided.

Drug Interactions

The drug interaction of salicylates are detailed in Table 1. Psoriatics receiving topical salicylates and methotrexates are at a potential risk of methotrexate toxicity.

Interference with Laboratory Tests

Salicylates may interfere with some laboratory tests as detailed in Table 2.

Table 2. Alterations of Laboratory Results During Salicylate Therapy

Test	Type of Abnormality and Mechanism
Thyroid Function ²²	Competes with thyroxine binding to TBPA. ↓ PBI. ↑ T ₃ uptake.
Urinary Sugar ²³	False negative with glucose oxidase. False positive with clinitest caused by gentisic acid metabolites in patients with 2-5 g salicylate q.d.
5-hydroxyindole acetic acid ²⁴	False negative when fluorometric test used
Acetone, ketone bodies	False positive FeCl ₃ in Gerhardt reaction. Reddish color persists with boiling.
17-OH Corticosteroids ¹⁸	False low with > 4.8 g q.d.
Vanilmandelic Acid ²⁴	False low.
Uric Acid ^{25*}	↑ or ↓ depending on dosage.
Prothrombin ^{26*}	↓ with > 2 gr q.d

* Physiological effect of drug.

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PERCUTANEOUS ABSORPTION OF SALICYLIC ACID
IN RABBITS

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The pharmacokinetic parameters defining the percutaneous absorption of salicylic acid in rabbits have been investigated. A one compartment open model with apparent first order absorption was found to describe adequately the blood level data.

INTRODUCTION

Topical application of salicylic acid preparations is a well established practice in dermatology, particularly for the long term treatment of psoriasis. Although the in-vitro release rate and the extent of percutaneous salicylic acid absorption in-vivo from various ointment bases has been thoroughly studied,¹⁻³ there is, as Taylor and Halprin⁴ and Cooper et al.⁵ have pointed out, a paucity of pharmacokinetic data characterizing the absorption of percutaneously applied salicylic acid. This paper reports estimates of the pharmacokinetic functions which define the topical absorption of salicylic acid in rabbits.

EXPERIMENTAL

Materials - Hydrophylic ointment U.S.P. (Ruger Chemical Co., Irvington, NJ), obtained commercially, was milled (Hammonia Ointment Mill, Josef Deckelmann, Aschaffenburg, W. Germany) to improve consistency and the ease of application. To provide a uniform particle size, salicylic acid (Fisher Scientific Co., Fairlawn, NJ), and urea (Fisher Scientific Co., Fairlawn, NJ), were passed through an 80 mesh sieve prior to use. All other chemicals were analytical reagent grade, and were used as received.

Salicylic acid in hydrophilic ointment (10% w/w) (I) and salicylic acid (10% w/w) and urea (10% w/w) in hydrophilic ointment (II) were prepared the day of application.

Tungstic acid reagent was prepared by mixing 10 ml. of 10% aqueous sodium tungstate solution and 80 ml. of 1/12 N sulfuric acid. The reagent was prepared fresh the day samples were analyzed.

Methods - Female New Zealand rabbits, age approximately five months and weighing between two and three kilograms, were housed in humidity and temperature controlled quarters and maintained on a diet of rabbit chow and water ad lib. Rabbits were placed in restraining cages for successively longer periods of time over a span of several days for conditioning before being used for an experiment.

The hair on the ventral side of the animal from the forelegs to the hindlegs was carefully removed with animal clippers two to three hours prior to application of a test vehicle, taking care not to cut or otherwise visibly damage the skin surface.

An intermittent infusion set, 21 gauge, with a 15.9 mm (5/8") needle (Butterfly-21, Intermittent Infusion Set, Abbott Laboratories, Chicago, IL) was inserted into the central ear artery, immediately prior to application of the test vehicle. The infusion line was filled with a dilute, 100 units per ml., heparin solution whenever samples were not being collected. This technique allows for repetitive sample collection without the need for numerous venipunctures. A blood sample was collected before application of the ointment to serve as a pretreatment control.

Ten grams of ointment was spread over a standard, rectangular, 7 cm. x 13 cm. (1.75 x 5.11") template following the technique of Stollar et al.¹ The template was applied to the shaved area of the animals abdomen and held in place with adhesive tape. The rabbit was then placed in a restraining cage for the duration of the experiment. Water was provided ad lib., food was given or withheld as reported in each study. Two ml. blood samples were taken, the serum separated and refrigerated at 4°C until assayed. Blood samples were taken immediately prior to application of a test vehicle, during the eight hour application period, and for eight hours following the removal of the vehicle template.

Total salicylic acid was assayed by the spectrofluorometric method of Saltzman.⁶ Nine and one-half ml. of tungstic acid reagent was added to 0.5 ml. of plasma, this solution was shaken and the reaction allowed to develop for at least ten minutes. The mixture was then filtered and 5 ml. of filtrate was added to 7 ml. of 10 N NaOH. The fluorescence of the resulting solutions was determined within thirty

minutes using a fluorometer (Turner Model 111, G. K. Turner Associates, Palo Alto, CA) fitted with a 405 nm primary filter and a secondary, sharp cut filter passing all light greater than 455 nm. Salicylic acid concentrations were determined by comparison to a standard curve. Plots of fluorescence as a function of standard salicylic acid concentrations were linear over the range 0.5 to 20 mg.% (correlation coefficient 0.99, $n = 8$).

RESULTS AND DISCUSSION

Salicylic acid plasma levels as a function of time for a fasted animal treated with (I) for eight hours is shown in Figure 1. Peak plasma salicylic acid concentrations observed were between 10 mg.% and 18 mg.%, and were attained within four to six hours of ointment application. It appeared that in most cases the absorption process was essentially completed prior to ointment removal. Plasma salicylic acid concentrations were detectable (greater than 0.5 mg.%) for at least twelve hours after the removal of the ointment.

The plasma salicylic acid concentrations in the post-absorptive phase appears to decline monoexponentially (Figure 1). The elimination rate constant, k_e , was calculated from a log-linear regression of the plasma salicylic acid concentration as a function of time. The elimination rate constant for eight rabbits treated with (I) for eight hours was determined to be $0.41 \text{ hr}^{-1} \pm 0.27 \text{ hr}^{-1}$ (s.d.) (Table I).

The Wagner-Nelson method is commonly used to characterize the absorption process.⁷ This technique is based on the one-compartment open model, and can be used to obtain data from which the order of

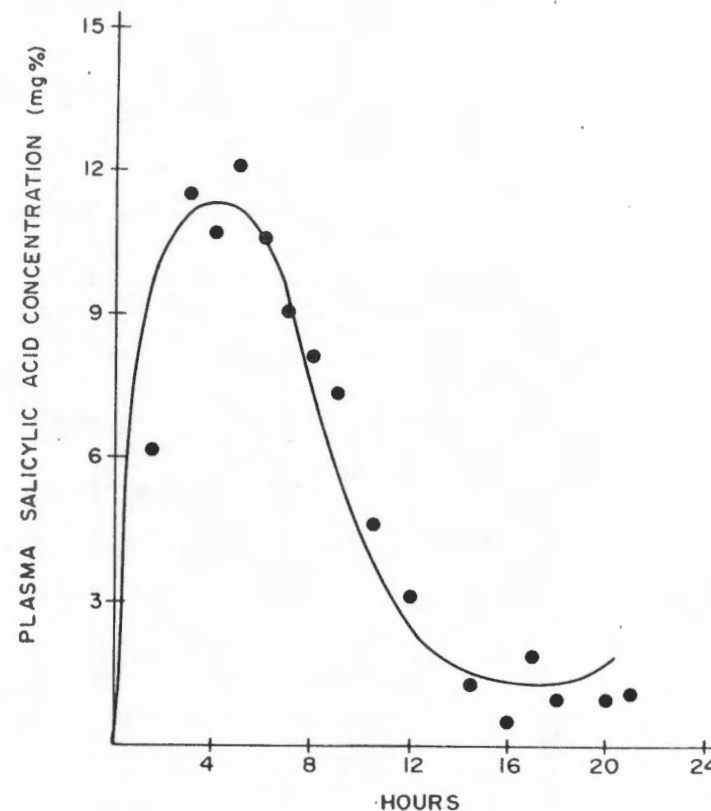


Figure 1

Plasma salicylic acid concentration as a function of time. Eight hour application of ointment (I).

absorption and the appropriate rate constant can be calculated. The Wagner-Nelson equation is

$$At/V = Ct + k_e \int_0^t C_p dt \quad (\text{Eq. 1.})$$

Where At/V is the amount of drug in the body at time t , divided by the volume of distribution V , C_t

TABLE I Pharmacokinetic parameters derived from the topical absorption of Salicylic Acid in the Rabbit

TREATMENT		
Ka	(hr ⁻¹ ± s.d.)	
Wagner-Nelson	0.34 ±	0.28
NONLIN	0.25 ±	0.07
Ke	(hr ⁻¹ ± s.d.)	
Elimination Plot	0.41 ±	0.27
NONLIN	0.29 ±	0.07
Lag Time	(hr ± s.d.)	
Hours	1.09 ±	0.16

represents the concentration of drug in the blood at time t , and the integral $k_e \int_0^t C_p dt$ represents the

cumulative amount of drug eliminated.

If the plot of the logarithm of the change in At/V versus time is linear, the absorption process can be characterized as first order and an absorption rate constant determined. A representative plot is shown in Figure 2. Such plots were linear in all cases

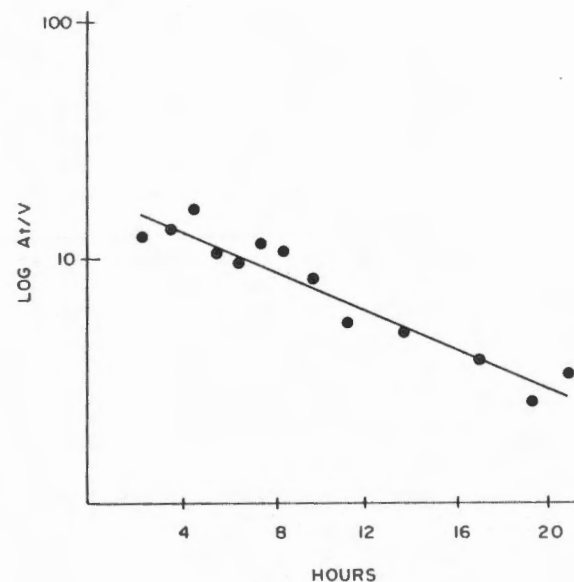


Figure 2

Absorption rate of salicylic acid calculated by the Wagner-Nelson method.

characterizing the absorption of topically applied salicylic acid as a first order process. An average absorption rate constant of $0.34 \text{ hr}^{-1} \pm 0.28 \text{ hr}^{-1}$ (s.d.) was obtained (Table I).

Since the absorption rate was characterized as first order, fitting the one compartment open model, the data was treated by a nonlinear least squares regression analysis program (NONLIN)⁸ to refine further the estimates of k_a and k_e . An appropriate set of equations for the one compartment open model with first order absorption was written for the IBM 370/155 digital computer. The parameters obtained graphically were used as initial estimates. Conver-

gence of parameters to a best fit occurred within 20 iterations for all data sets, and the regression coefficients were greater than 0.9. The values of k_a and k_e calculated directly by graphic methods previously described and by NONLIN (Table I) were not found to be statistically different (Student's t test, $p = 0.05$), most likely due to the large standard deviations in absorption and elimination rates.

A lag time, defined as the time required for the absorption process to become pseudo first order, was needed to describe the percutaneous absorption of salicylic acid. By expressing the amount of drug absorbed at time t as a percentage of the total amount absorbed (A/V) and subtracting from 100 the percent remaining to be absorbed can be calculated. A plot of the percent remaining to be absorbed as a function of time was linear with a slope equal to k_a (Figure 3). Performing linear regression analysis and solving for the time at which 100% of drug remains to be absorbed yields the lag time. A value of $1.09 \text{ hr} \pm 0.16 \text{ hr}$ (s.d.) was obtained (Table I).

In contrast to previous studies in which animals were fasted throughout an experiment, two animals were fed approximately 30 grams of rabbit chow fifteen hours following the application of ointment (I). A plot of plasma salicylic acid concentration as a function of time for an animal fed is shown in Figure 4. Absorption and elimination rate constants calculated from the plasma salicylic acid concentrations through the first sixteen hours are not significantly different from those calculated using fasted animals. However, whereas in fasted animals plasma salicylic acid levels remained low, less than 2 mg%, for the remainder of the collection period (Figure 1), a

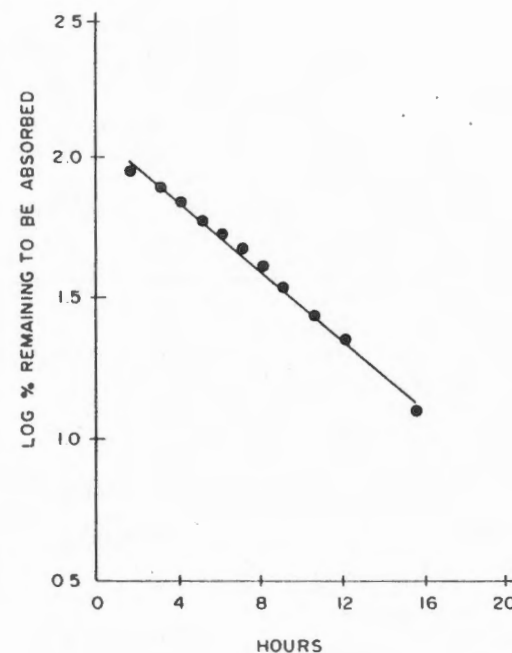


Figure 3

Percent salicylic acid remaining to be absorbed.

second peak was observed in both the animals fed during the collection period. In both cases the plasma concentrations prior to feeding were consistent with those observed in fasted animals. Secondary peak concentrations above 6.5 mg% were detected 3 to 5 hours following the feeding. These peaks correspond to times approximately nine and twelve hours after the removal of the ointment. As a result, it is unlikely that the second peak can be attributed to additional absorption of salicylic acid through the skin. This peak seen only in animals fed during an experiment may perhaps be attributable to biliary recycling.

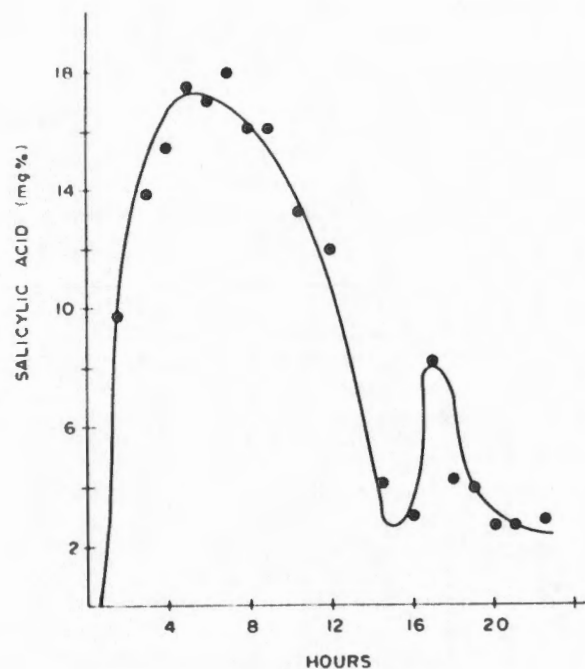


Figure 4

The effect of feeding on the plasma salicylic acid concentration time profile following the topical administration of salicylic acid.

Recycling of drugs excreted into the bile in unchanged form and for metabolites is possible, if they are either absorbed or modified by the gut flora, particularly after splitting of conjugates.⁹ The form of drug which is excreted into the bile also affects recycling, drug which form glucuronides have been shown to undergo recycling, since the glucuronide can be readily cleaved to form the free drug in the bile. Salicylic acid in man is metabolized by the liver, and its metabolites, salicylacylglucuronide,

salicylphenolicglucuronide, salicyluric acid, and gentisic acid are found in the bile.¹⁰ If the unchanged drug and the glucuronide account for the greater portion of the drug in the bile of rabbits as in man, it is quite likely that biliary recycling occurs.

The results of biliary recycling of drugs is commonly seen as either a series of irregularly spaced peaks in the declining portion of a blood concentration as a function of time plot or simply as a prolonged elimination phase.⁹ Since the rabbit, unlike some species, is known to store bile and release it upon stimulation such as food,¹¹ it is possible that the stimulus provided by the small amount of food caused the release of bile which in turn led to the reabsorption of salicylic acid.

Studies designed to investigate the possible utility of urea to enhance percutaneous absorption did not reveal any effect on the rate of absorption of topically applied salicylic acid.

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MOISTURE, HARDNESS, DISINTEGRATION AND DISSOLUTION
INTERRELATIONSHIPS IN COMPRESSED TABLETS PREPARED
BY THE WET GRANULATION PROCESS

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ABSTRACT

Interrelationships among moisture, hardness, disintegration and dissolution in compressed tablets were studied by compressing tablets from granulations prepared by the wet granulation process containing low moisture levels. Hardness, disintegration and dissolution of these tablets did not change on exposure to ambient room conditions. After equilibration under high humidities, a decrease in tablet hardness occurred which depended linearly on tablet hardnesses at the time of compression. After overnight exposure to ambient room conditions, the softened tablets increased in hardness and this increase greatly exceeded the initial hardnesses. The magnitude of hardness increase was independent of the hard-

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Percutaneous Absorption of Salicylic Acid

J. Richard Taylor, MD,
Kenneth M. Halprin, MD

The potential hazards of repeated topical application of salicylic acid under occlusion to large areas of the body was evaluated by measuring the percutaneous absorption and serum salicylate concentrations in four patients with active psoriasis.

Serum salicylate concentrations never exceeded 5 mg/100 ml in any of the patients, and although greater than 60% of the salicylic acid applied was absorbed, no evidence of accumulation or toxicity was observed. This form of treatment appears to present little potential hazard even in patients with extensive skin disease. Therapy could be hazardous for patients with impaired hepatic or renal function or for smaller children.

The urinary excretory products of salicylate metabolism were compared following topical and intravenous salicylate administration to determine if the skin plays any part in the biotransformation of salicylate during percutaneous absorption. Our data are too limited and inconclusive to answer this question.

Topical preparations containing salicylic acid have been used in dermatology for many years, and salicylate toxicity resulting from their use has been reported.¹⁻³ Unfortunately there is little information available concerning the potential

hazards of using large quantities of salicylic acid on large areas of the body surface in situations where skin disease exists and occlusive therapy may be desirable. A preparation containing 6% salicylic acid in a gel comprised of 60% propylene glycol and 19.4% alcohol (Keralyt) has recently become available for the treatment of patients with generalized skin disease under occlusion. This study is an attempt to evaluate the potential hazard of using this product. This was accomplished by determining the percutaneous absorption and serum salicylate levels produced when this preparation is used in a well-controlled clinical setting designed to provide maximal exposure. To determine if the degradation of salicylic acid is altered when salicylate is absorbed percutaneously, the products of salicylate metabolism excreted in the urine were compared after topical and intravenous administration.

PATIENTS AND METHODS

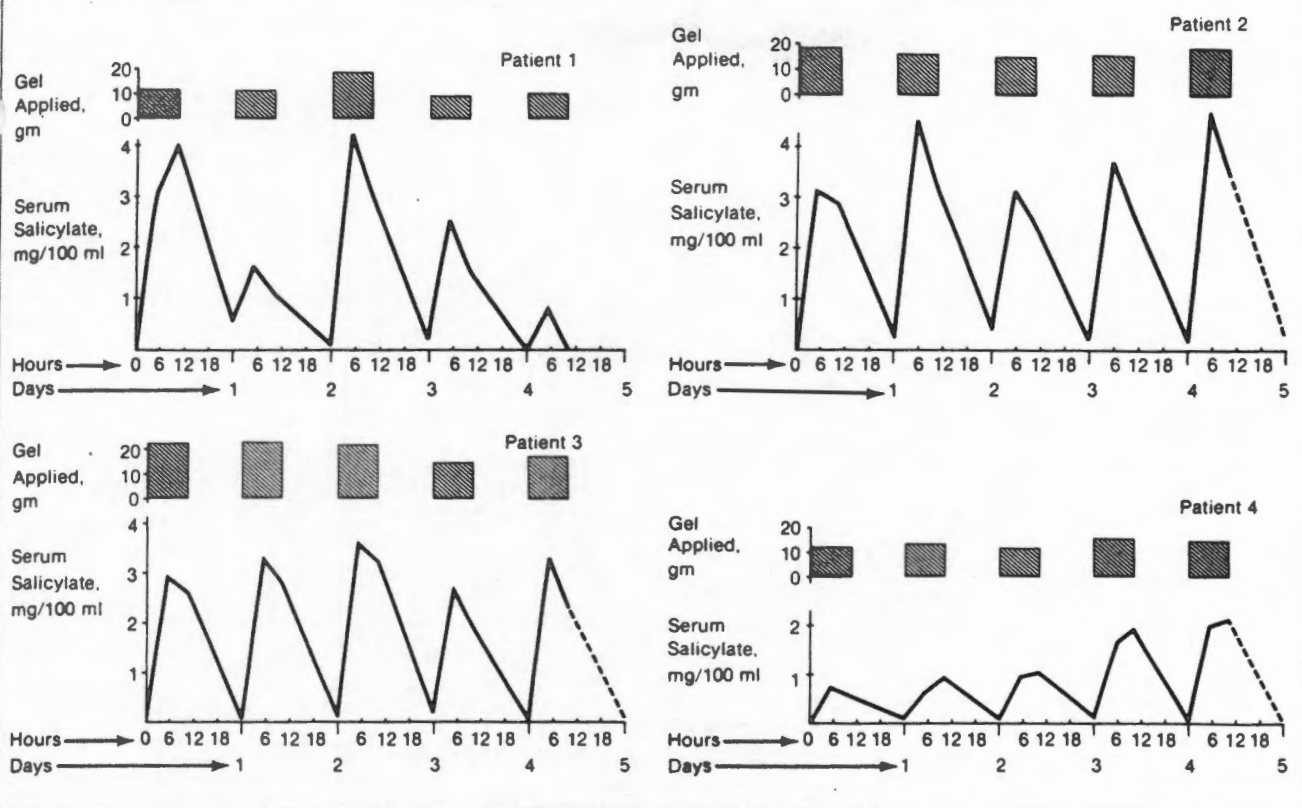
Patients

Four patients with active psoriasis were studied as inpatients at the Miami Veterans Administration Hospital. None of the patients had renal disease, had ingested salicylate-containing medications for one week prior to the study, or was permitted to use other topical or systemic medications for the duration of the study. All of the patients had more than 25% of their body surface involved with psoriasis (patient 2 had severe generalized psoriasis, with more than 90% involvement). In-

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Serum salicylate concentrations and the number of grams of 6% salicylic acid gel applied for days 1 through 5 for each of the four patients are shown. Dotted line connects point representing last salicylate level which was measured (tenth hour of day 5) to base line for illustrative purposes.

formed consent was obtained from each patient after the procedures and hazards of the study had been fully explained.

Patients applied the contents of a 28-gm tube of medication to their entire body surface below the neck immediately after taking a shower. The treated areas were covered with plastic wrap and kept occluded for ten hours. After ten hours, the dressings were removed and the patient allowed to shower. This treatment was repeated daily for a total of five days. All tubes of medication were preweighed and then reweighed after use to determine the grams of gel applied by each patient each day.

One week after completing the above study patient #3 was given 1 liter of physiological saline solution containing 1.0 gm of sodium salicylate intravenously during an eight-hour period.

Collection of Samples

Venous blood was obtained prior to application of the topical medication and five and ten hours after application each day for five days. The serum was separated and stored refrigerated until analyzed.

All of each patient's urine was collected for each 24-hour period starting from the

beginning of the study for a total of seven days. At the end of each day, the urine volume excreted was measured; an aliquot was sent to the hospital clinical laboratory for creatinine determination, and an aliquot was refrigerated for analysis of salicylates.

Serum was obtained in a similar manner from the patient receiving intravenous sodium salicylate, but it was collected at 0, 4, 8, and 24 hours after injection. Urine was collected prior to and for three days following the intravenous administration.

Salicylate Determinations

The serum salicylate level was determined fluorometrically by the method described by Saltzman.⁴

Salicylic acid and its metabolites in urine were determined by the method of Levy and Procknal.⁵ This procedure determines the total grams of salicylate excreted and the percent of this total present as free salicylic acid, salicyluric acid, and the acyl and phenolic glucuronides of salicylate.

RESULTS

The Figure illustrates the serum salicylate concentrations and grams of

gel applied for each of the four patients for the five days that serum samples were collected. In no instance did the serum salicylate concentration exceed 5 mg/100 ml, and the average peak serum concentration for all of the patients was less than 3 mg/100 ml (2.7). The patient with the most severe psoriasis (patient 2) developed the highest serum salicylate concentrations (Figure). The serum salicylate levels had returned to low or undetectable values by 24 hours after administration of the drug, and in no instance did there appear to be an accumulation of salicylate during the five days of treatment. A gradual accumulation of salicylate might be suspected in patient 4 (Figure) because of the progressive increase in his serum salicylate levels, but this was no doubt the result of applying greater quantities of topical medication since his serum levels returned to normal prior to each subsequent day's treatment.

The results of the urine salicylate

analyses are summarized in Table 1. We were able to recover more than 60% of the salicylic acid applied topically in the urine of all four patients. Patient 2 had the greatest percutaneous absorption; but as indicated previously, he also had the most widespread psoriasis. These results indicate that salicylic acid is efficiently released from the vehicle we used, and it encounters very little resistance to penetration through diseased skin under occlusion.

Table 1 also shows the percent of the total salicylate excreted, which is represented by each of the three major metabolites. In table 2, the percent of the total salicylate excreted as each metabolite after topical application of salicylic acid gel is compared to the metabolites found following intravenous sodium salicylate administration for patient 3. These percentages were calculated from the total quantities of each metabolite excreted during the period of the study and not by averaging the percentages of each excreted per day. We have also included in Table 2 the expected percent of each of the salicylate metabolites after oral administration, as reported in the literature,⁸ for purposes of comparison. All of our patients excreted less salicylate as salicyluric acid and more as salicylate glucuronides than expected. Patient 3 exhibited these changes not only following topical application of salicylic acid but also when given sodium salicylate intravenously.

COMMENT

The findings of our study indicate that salicylic acid is rapidly absorbed when applied in a propylene glycol-alcohol gel vehicle under occlusion to diseased skin. When applied under occlusion for ten hours, more than 60% of the salicylic acid applied was absorbed. In three of the four patients, peak serum levels occurred within five hours, despite the fact that occlusion was carried out for ten hours. The serum salicylate concentration never exceeded 5 mg/100 ml, however, which is well below levels of 30 to 40 mg/100 ml associated with systemic toxic reactions.⁹ Since 28 gm of this preparation will easily cover the

Table 1.—Results of Urinary Salicylate Analyses*

Day	Gel Applied, gm	SA Applied, mg	Salicylate Excreted, mg	Urinary Metabolites			
				% SA	% SU	% SG	
Patient 1							
1	12.4	744	634	4	58	38	
2	11.6	696	369	3	53	44	
3	18.3	1098	584	3	57	40	
4	9.4	564	400	2	62	36	
5	10.1	606	309	3	65	32	
6	0	0	69	3	69	28	
7	0	0	5	18	47	35	
Total	61.8	3708	2370				
Absorption: 64%							
Patient 2							
1	18.5	1110	690	14	52	34	
2	16.0	960	848	11	53	36	
3	14.7	882	750	13	54	33	
4	15.3	918	690	4	55	41	
5	18.8	1128	874	4	48	48	
6	0	0	7	—	—	—	
7	0	0	213	1	62	37	
Total	83.3	4998	4072				
Absorption: 82%							
Patient 3							
1	22.3	1338	615	8	58	34	
2	22.6	1356	684	6	56	38	
3	21.8	1308	764	7	52	41	
4	14.4	864	1123	2	41	57	
5	17.2	1032	553	6	54	40	
6	0	0	1	—	—	—	
7	0	0	0	—	—	—	
Total	98.3	5898	3740				
Absorption: 63%							
Patient 4							
1	12.1	726	210	5	57	38	
2	13.5	810	349	0	60	40	
3	12.1	726	326	3	57	40	
4	15.9	954	605	6	56	38	
5	14.8	888	927	4	51	45	
6	0	0	410	3	53	44	
7	0	0	0	—	—	—	
Total	68.4	4104	2827				
Absorption: 69%							

* SA indicates salicylic acid; SU, salicyluric acid; SG, acyl and phenolic glucuronides of salicylic acid; — indicates concentration insufficient for reliable determination.

Table 2.—Salicylate Metabolites Excreted After Different Routes of Administration

Route of Administration	% of Total Urinary Salicylate		
	Free Salicylic Acid	Salicyluric Acid	Salicylate Glucuronides
Topically applied gel containing salicylic acid	6	52	42
Intravenously administered sodium salicylate	3	63	34
Orally administered salicylate*	10	70	20

* Data obtained from Goodman and Gilman.⁸

entire body surface of a large man and occlusive therapy would rarely be used for more than ten hours each day, this form of treatment appears to present little potential hazard even in patients with extensive skin disease.

Absorbed salicylic acid distributes into the extracellular fluid volume (150 ml/kg),⁸ which is about 10 liters in a 72-kg man. A 28-gm tube of 6% salicylic acid contains 1.8 gm of salicylic acid; if 60% of it is absorbed, the maximal serum level that could result would be about 1 gm/10 liters or 10 mg/100 ml, which is well below toxic concentrations. Toxic reactions would not have occurred in this study even if 100% of the applied salicylic acid had been absorbed since the expected serum level (18 mg/100 ml) would still be below a toxic level. We did not believe it was necessary to produce a toxic reaction to salicylate to adequately determine the percutaneous penetration of salicylic acid, and the study was designed to minimize this possibility.

Topical salicylic acid therapy can be hazardous, however, and salicylate toxicity as well as death from acute salicylate poisoning can occur from its injudicious use. To prevent a toxic reaction, three factors should be kept in mind. The quantity of salicylic acid used should not be excessive, the available extracellular fluid volume should not be limited, and the ability to metabolize and excrete the absorbed medication should not be im-

paired. Excessive quantities of salicylic acid can result from either a high concentration in the medication or from frequently repeated applications to large areas of the body. The two fatal cases reported by Lindsey⁴ occurred after only two applications of an antifungal solution containing 20.7% salicylic acid to 50% of the body surface area. vonWeiss and Lever³ reported a toxic reaction to salicylate in three patients treated with 3% and 6% salicylic acid ointment applied six times daily for 4 to 11 days.

Toxic reactions to salicylate occur more frequently in smaller children because their extracellular fluid volume is much smaller in comparison to the potential surface area available for treatment. A contracted extracellular volume is also a potential hazard in dehydrated debilitated patients. Such patients can also develop a toxic reaction to salicylate as a result of impaired excretion if hepatic or renal disease exists. These factors may have contributed to the toxic reaction to salicylate reported in some cases,^{2,3} although they were not specifically discussed. The cases reviewed by Young,¹ however, demonstrate the increased risk of treating children.

Most of the salicylic acid absorbed is metabolized to more water soluble products to facilitate its excretion. Salicylic acid is conjugated with glycine, forming salicyluric acid, which normally accounts for 70% of the salicylate excreted. It is also conjugated with glucuronic acid forming several

salicylate glucuronides, which as a group account for 20%; the remainder is excreted as free salicylic acid. Although the metabolism of salicylate occurs primarily in the liver, the intestinal mucosa can also synthesize salicylate glucuronides.¹⁰ We wondered if the skin might also possess the glucuronyl transferase system necessary for this conjugation. This would explain the higher than expected urinary salicylate glucuronide levels found in our patients. To further study this possibility, we administered salicylate intravenously in one of our patients to see what percent of the excreted salicylate was salicylate glucuronide when its absorption did not occur through either the intestinal mucosa or skin.

We expected that intravenous administration, which would by-pass possible conjugation systems in both the gut and the skin, would result in a lower level of glucuronides than after topical (or oral) administration. Table 2 shows that the amount of salicylate excreted as glucuronides was lower than after topical application, but it was still fairly high and leaves open the question of the possible glucuronide conjugation of drugs by the skin. This question should be approached by more direct biochemical methods for the demonstration of the presence and in vivo activity of this drug metabolizing system.

The salicylic acid used in this investigation was supplied as Keralyt by Westwood Pharmaceuticals Inc., Buffalo, NY.

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Percutaneous Absorption of Salicylic Acid after Repeated (14-day) *In Vivo* Administration to Normal, Acneogenic or Aged Human Skin

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Abstract □ The objective of the present work was to determine the relative bioavailability of salicylic acid (SA) after repeated (14-day) topical application to subjects who presented normal, acneogenic, or photodamaged facial skin. To emulate exposure characteristics likely to be encountered by subjects in these two subpopulations, individuals presenting facial acne were treated with 2% SA in a hydroalcoholic vehicle, and volunteers with aged or photodamaged skin received a comparable topical dose of SA in a cream (moisturizer-like) vehicle. Plasma concentration-time profiles and cumulative urinary excretion of SA were measured after the last dose in subjects who had received 15 consecutive daily topical applications of 27 mg of SA or oral doses of 81 mg of acetylsalicylic acid (ASA). The rate and extent of percutaneous absorption of SA were not affected by facial skin condition. Faster rates of absorption (C_{max}) were obtained with a hydroalcoholic compared with a cream vehicle. Systemic SA exposures were at least five-fold higher with oral ASA than topical SA. Based on systemic salicylate concentrations resulting from ingestion of 81 mg of ASA, these results support that patients without gross skin disorders are at minimal risk of adverse systemic effects from routine use of topical products containing 2% SA.

Introduction

Salicylic acid (SA) is widely used as a topical therapeutic agent for a variety of skin diseases, including acne, psoriasis, and ichthyosis. As an over-the-counter (OTC) topical medication, SA is approved at concentrations up to 2% (w/w) by the FDA Final Acne Monograph.¹ Numerous studies have demonstrated that following deposition onto the skin, SA will penetrate the stratum corneum and enter the systemic circulation.²⁻⁴ Topical application of 6% SA ointments to large body surface areas for the treatment of psoriasis and ichthyosis has been associated with high plasma concentrations of salicylates and even sporadic reports of clinical toxicities.^{5,6} To our knowledge there have not been any reports associating topical use of 2% SA with these adverse systemic effects; however, as the number of products containing 2% SA increases in the marketplace, there is a need to better define cutaneous SA absorption from these preparations.

The primary objective of this study was to determine plasma salicylate levels in human subjects following application of 2% SA in two different vehicles as a function of facial skin condition. To this end, the study design allowed subject group designation to vary by both skin type and SA application vehicle. SA bioavailability from a hydroalcoholic vehicle was evaluated after topical application in subjects with normal facial skin and subjects with dermatologically confirmed mild to moderate facial acne. SA bioavailability from a cream vehicle was evaluated in subjects with normal facial skin and subjects with medically confirmed evidence of moderate to very severely aged and/or photodamaged facial skin. A fifth

subject group that received 81 mg of aspirin (acetylsalicylic acid; ASA) daily rather than daily SA skin treatment was included as a reference control.

Materials and Methods

Subjects—Thirty-eight female volunteers, 18 to 65 years of age, were assigned to four treatment groups based on dermatologically assessed facial skin characteristics: two groups of subjects presented normal skin, one group presented mild to moderate acne, a fourth group was selected for evidence of moderate to severely aged or photodamaged skin, and a fifth group, which served as the reference control, was required to meet no facial skin criteria. Overall, no subjects in the five treatment groups had active skin disease, scarring, or excessive hair formation. Furthermore, for normal skin designation, subjects had no apparent acne and an aged or photodamaged designation of mild or less. For aged or photodamaged designation, subjects had no apparent acne, and presented with at least moderate facial wrinkling, and/or uneven texture or tone (color). The degree of photodamage was assessed with a photographic comparison scale that allowed the investigator to select 1 of 6 designations from mild to extremely severe. The investigator who performed these assessments had been previously familiarized with this scale, which is similar to that used by Bhawan et al.⁷ For acne group designation, an overall acne grade of 3 - 9 according to the global acne scale, combined with a full facial lesion count of at least 15 open or closed non-inflamed comedones and at least 15 inflamed papules and/or pustules was required. The global scale used was modified from Burke and Cunliffe.⁸

Only female subjects were included in the study because some of the more concerning toxicological effects of salicylates are in the areas of maternal reproductive and fetal toxicity. All subjects were determined to be in good health by general medical and clinical chemistry evaluations, which included blood chemistry, routine hematology and serology, urinalysis, urine pregnancy test, and drug screen. Subjects selected were not on any OTC medications, prescription drugs, or oral contraceptives and were either surgically sterile, postmenopausal, or using an acceptable barrier method of contraception.

Subjects were instructed to avoid salicylates in oral and topical medications, as well as other potential sources of salicylates (e.g., wintergreen oil) for at least 1 week prior to the start of the study and throughout the duration of the study. Only nonsmokers and subjects without a history of drug and alcohol abuse were included in the study. Subjects were also instructed to refrain from the intake of alcohol or caffeine during the study and for at least 72 h prior to study start.

Study Design—This study received institutional review board approval, and written informed consent was obtained from each volunteer prior to her entering the study. The study was designed to evaluate serum salicylate concentration-time profiles in female subjects after topical application of 2% SA. SA was applied in either a cream or hydroalcoholic liquid to the subjects' face and neck. Serum salicylate concentration-time profiles also were compared with female subjects who received oral administration of acetylsalicylic acid (ASA). Subjects were divided into five groups of 9 to 10 individuals; four groups were based on skin type (aged, acneogenic, and two normal groups) as already defined, and one group was based on administration route (oral).

Subjects who received topical SA were designated by both skin type and application vehicle. Nineteen subjects with normal skin (normal skin subjects) were enrolled in the study. Ten of these subjects were

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treated with the 2% SA cream. The remaining nine normal skin subjects were treated with the 2% SA hydroalcoholic liquid. Nine subjects with aged (photodamaged) skin (aged skin subjects) were enrolled in the study and treated with the 2% SA cream and nine subjects with acne (acne skin subjects) were treated with the hydroalcoholic liquid. All facial applications of SA were performed once daily at the study site for 16 days by trained study personnel. This treatment interval was selected on the basis of known skin cell cycle dynamics to allow for the expression of any potential application-related effects on skin barrier function that could possibly impact SA percutaneous absorption.⁹ The amount of test material applied was ~1.25–1.50 g (25–30 mg SA), and the material was applied to the face and neck only. Subjects in the oral aspirin reference group came to the clinical site daily to receive 81 mg of ASA with 8 ounces of water once daily. On day 15 of the study, all subjects were confined to the testing facility for 24 h. Blood samples were taken periodically for pharmacokinetic analyses. Total urine was collected to determine salicylate excretion.

Test Materials—Two different SA application vehicles were included in this study: a hydroalcoholic liquid containing 63% water, 35% ethanol, and 2% salicylic acid; and a cream consisting of 80% water, 2% salicylic acid, and 18% cosmetic excipient mixture (PPG-14 butyl ether, glycerin, cetyl and stearyl alcohols, polyquaternium 37, mineral oil, dimethicone, Steareth-21, cyclomethicone, and triethanolamine). Bayer Children's Aspirin (lot #KD340) was purchased commercially (81 mg of ASA per tablet).

Blood and Urine Sample Collection—Blood samples (7 mL in EDTA/Na fluoride vacutainers) were obtained by venipuncture of the forearm on study days 0, 7, and 12. All subjects were sequestered at the clinical site for a 24-h period on study day 15 and provided with three controlled meals throughout the day. Upon arrival to the facility, each subject was instrumented with an indwelling forearm venous catheter to obtain a pre-dose blood sample, as well as post-dose samples at 5, 15, 30, and 45 min, and 1, 2, 3, 4, 6, 8, 10, 12, 16, and 24 h. After collection, the samples were centrifuged at 3000 rpm for 10 min, and plasma was frozen at -70 °C until assayed for salicylate content. For each subject, urine was collected and kept under refrigeration throughout the collection period. At the completion of the 24-h collection period, each urine sample was weighed and an aliquot was maintained frozen at -70 °C until assayed for salicylate content.

Analysis of Salicylate in Blood and Urine—ASA (plasma), SA (plasma), and total salicylate concentrations (urine) were determined by high-pressure liquid chromatography (HPLC) with a procedure modified from Buskin et al.¹⁰ and Siebert and Bochner.¹¹ For the urine assay, the sample (one part urine) was first diluted in a 1:1.5 parts water:concentrated hydrochloric acid mixture and hydrolyzed by autoclaving at 120 °C for 60 min to convert salicylate metabolites (mainly salicylic acid) to SA. The urine sample prepared in this manner was then subjected to the same analysis routine as used for plasma ASA and SA, which involved acidification, extraction from plasma with ether/hexane, back extraction with phosphate buffer, and determination by HPLC. Tandem UV and fluorescence detection allowed simultaneous determination of ASA and SA. ASA was determined with a Kontron 432 UV detector (absorption at 234 nm), and SA was determined by fluorescence with a Hitachi F 1060 (excitation, 300 nm; emission, 433 nm). The ASA method quantification limit was 20 µg/L, and the SA quantification limit was 5 µg/L. Standard curves were linear over ranges of 20 to 1000 and 5–1000 µg/L, respectively, for ASA and SA. Percent recovery for plasma SA, as determined with samples spiked with SA at 200 µg/L, was 97 ± 6% (mean ± standard deviation; *n* = 17). For urinary SA, percent recoveries of samples spiked with 1:1 mixtures of SA:salicylic acid (*n* = 8–10) were 88 ± 6% (100 µg/L), 88 ± 2% (200 µg/L), and 84 ± 1% (500 µg/L).

Data Evaluation and Statistical Analysis—The maximum plasma concentration (C_{max}) of SA and the time at which the maximum concentration occurred (T_{max}) were determined from the individual SA plasma concentration–time profiles. The terminal exponential half-life ($t_{1/2}$) was estimated from linear least-squares regression of the terminal phase of the log concentration–time profile following the last dose. Area under the plasma concentration–time curve (AUC) was determined by the linear trapezoidal rule.^{12,13}

To determine the time to reach steady-state, trough plasma SA concentrations at days 7, 12, 15, and 16 were analyzed by one-way ANOVA, which included

Table 1—Demographic Data for Study Participants^a

Skin Type	Age, Years (Range)	Weight, kg (Range)	Height, cm (Range)
Normal skin/hydroalcoholic vehicle (<i>n</i> = 10)	29.4 ± 7.62 (19–42)	62.5 ± 10.3 (50–78.4)	160.9 ± 7.71 (149.9–172.3)
Normal skin/cream vehicle (<i>n</i> = 10)	34.3 ± 10.3 (19–44)	65.6 ± 10.0 (50–79.5)	162.3 ± 7.49 (150–172.7)
Acneogenic (<i>n</i> = 9)	28.4 ± 5.4 (22–38)	68.0 ± 12.2 (50.5–86.8)	169.1 ± 10.6 (150–186)
Aged (<i>n</i> = 10)	52.0 ± 7.9 (38–62)	65.9 ± 9.3 (45.9–78.4)	162.1 ± 6.2 (152–170)
N/A ^c (<i>n</i> = 10)	38.1 ± 7.9 (24–47)	68.8 ± 12.3 (48.5–83.0)	162.6 ± 5.9 (152–170)

^a Subject demographics were determined at study enrollment. As described in Results, three of the subjects included in Table 1 were dropped from data analysis. Data presented are mean ± SD. The range for each observation is given in parentheses. ^b Race was ~20% black and 80% Caucasian for normal skin and oral ASA groups and 100% Caucasian for acneogenic and aged skin groups. ^c N/A, Not applicable. These subjects were assigned to receive oral ASA and therefore were not required to meet a facial skin criterion.

factors for skin type (normal, acneogenic, aged). The treatment by skin type interaction term was included to test for differential effects over time as a function of skin type. Pharmacokinetic parameters (C_{max} , T_{max} , $t_{1/2}$, and AUC) were analyzed by one-way independent measures ANOVA. As appropriate, all post hoc pairwise comparisons were done by the Student-Newman-Keuls multiple comparison procedure.¹⁴

To calculate the relative bioavailability (F_{rel}) of SA following topical administration versus oral administration, the following equation was used: $F_{rel} (\%) = [AUC (topical)/AUC (oral)] \times [dose (oral)/dose (topical)]$.

Results

Subject demographics are presented in Table 1. There were no statistical differences in the weight and height variations between the treatment groups. Subjects classified with aged or photodamaged skin (average age of group was 51 years) were significantly older than either of the two groups presenting normal skin or the subjects with acne.

All but one subject completed the study. Subject 10 was dropped because of failure to return to the study site for daily administration of test material. A second subject (subject 30) was excluded from data analysis because of abnormally high baseline levels of salicylates on study day 0, suggesting noncompliance with the self-medication requirements for the study. Subject 45 was also excluded from data analysis because of abnormally high baseline concentrations of SA on study day 15, suggesting similar noncompliance. There was no clinically observed skin irritation as a result of test material application in any of the study subjects, nor did subjects receiving oral aspirin report any adverse reactions. The average SA dose administered on day 15 of the study for the topically treated subject groups was 27 ± 0.8 mg. Importantly, there were no differences in the total amount of SA applied to the skin between topical treatment groups. Because topical preparations were applied daily by the same trained personnel, we would anticipate that daily doses across the study were at or about 27 mg.

Prior to administration of topical or oral salicylate, trough blood samples were collected on study days 7, 12, 15, and 16 to determine steady-state levels (Table 2). These results indicate that steady state was reached by day 7.

The plasma concentration–time profiles for SA after topical administration on the 15th day are illustrated in Figure 1. The corresponding pharmacokinetic parameters are shown in Table 3. Both the SA plasma concentration–time profiles and extent of percutaneous absorption of SA were influenced by the vehicle. Peak plasma SA concentrations were significantly

Table 2—Trough Plasma Salicylate Concentrations in Subjects Treated Daily with Topical or Oral SA*

Facial Skin	Vehicle	Plasma Salicylate Levels ($\mu\text{g/L}$)			
		Day 7	Day 12	Day 15	Day 16
Normal	Cream	36.10 \pm 21.29 ^a	14.40 \pm 2.23	23.70 \pm 8.06	29.50 \pm 6.99
Normal	Hydroalcohol	52.11 \pm 13.08	44.00 \pm 8.27	40.33 \pm 7.86	41.67 \pm 6.47
Aged	Cream	36.44 \pm 5.84	36.11 \pm 9.89	29.33 \pm 11.63	21.56 \pm 4.67
Acnegenic	Hydroalcohol	60.78 \pm 21.29	43.56 \pm 13.06	35.56 \pm 9.93	34.56 \pm 6.43
N/A	Oral aspirin ^b	<10.0 ^c	<5.0	<10.0	<10.0

* Data presented are the mean \pm SEM of $n = 10$ subjects in the normal/cream group and $n = 9$ in the remaining groups. ^a N/A, Not Applicable. Subjects scheduled for oral ASA administration were not required to meet a facial skin criterion. ^b All subjects had concentrations less than level indicated, and most had nondetectable concentrations.

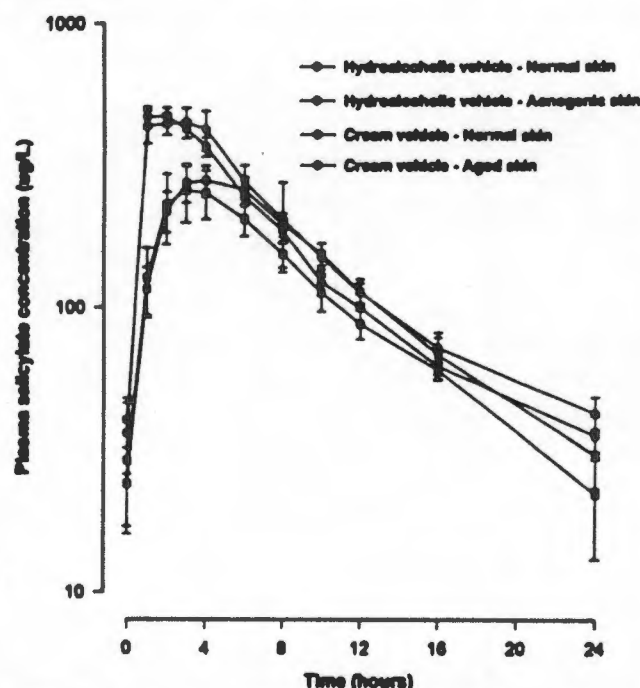


Figure 1—Plasma concentration-time profiles of SA in subjects presenting normal, aged, and acnegenic facial skin, after topical (face/neck) administration of a hydroalcoholic or cream vehicle containing 2% (w/v) SA. The data presented are the mean \pm SEM for $n = 10$ (normal/cream group) or $n = 9$ (all other groups). Where not shown, the SEM bar was contained within the corresponding symbol. For clarity, plasma SA levels measured at time points between 0 and 1 h are not shown in the figure.

higher and time to peak occurred earlier in subject groups that received 2% SA in hydroalcoholic vehicle as compared with 2% SA in the cream vehicle. There was a statistically significant effect of treatment on salicylate terminal exponential half-life as determined by one-way analysis of variance ($F_{4,46} = 6.57; p < 0.0003$). The SA terminal exponential half-life among subjects receiving oral ASA was significantly shorter than that observed in each of the topical treatment groups ($p < 0.05$). No significant pairwise differences in the terminal exponential half-life was seen among any of the groups treated with topical SA (i.e., no significant skin or vehicle effects by pairwise comparisons). AUC SA values were significantly higher in hydroalcoholic SA-treated subjects than in the cream-treated counterparts (Table 3). Skin type did not significantly influence any of the kinetic parameters examined.

The SA plasma concentration-time profile for subjects who received 81 mg of ASA is provided in Figure 2. For the subjects in this treatment group, the average time to peak plasma concentration was 0.71 h, with the average peak plasma SA concentration being 5283 $\mu\text{g/L}$. The corresponding plasma AUC value for this group was 22 010 $\mu\text{g h/L}$. Plasma

concentrations of the parent compound, ASA, were also measured in this study (data not given). Peak ASA levels were achieved ~30 min after oral administration and then declined rapidly with no detectable ASA in the plasma at 3 h post-administration. Peak concentrations of ASA (30 min) ranged from 80 to 300 $\mu\text{g/L}$. Peak and AUC concentrations of SA were significantly greater (5- to 10-fold) for the oral ASA group than the subjects receiving SA via topical administration.

Cumulative 24-h urinary SA excretion is shown in Table 4. No statistically significant pairwise differences in cumulative urinary levels of SA were seen among any of the groups treated with topical SA (i.e., no significant skin or vehicle effects by pairwise comparisons). Urinary recovery was significantly higher ($p < 0.05$) in the oral ASA group compared with each of the topical SA treatment groups.

Discussion

In general, SA is considered to penetrate well across human and animal skin.^{2,4,12,16} However, percutaneous absorption of SA is influenced to a significant extent by variables such as concentration, type of vehicle used for solubilization, skin hydration, and barrier condition.²⁻⁴ As a result, estimating systemic exposure to SA from use of commercial 2% SA skin care products is difficult given the existing literature. This study design allowed for quantification of the relative SA dermal absorption from two skin application vehicles (hydroalcoholic liquid and cream). Acnegenic skin subjects were treated with the hydroalcoholic vehicle because SA acne products often utilize alcohol-based matrices, and photodamaged or aged skin subjects were treated with the cream vehicle because cosmetic products containing SA most often utilize cream-based matrices.

Penetration of SA across the skin occurred readily following its application in either the hydroalcoholic liquid or the cream. However, the nature of the vehicle influenced both the rate and extent of absorption, with the hydroalcoholic vehicle allowing for higher percutaneous absorption. Although a comparative finding such as this has not previously been reported, prior studies have demonstrated significant percutaneous absorption of SA from alcohol based vehicles.¹⁷⁻¹⁹ Interestingly, in our studies, the SA plasma concentration-time profile did not differ significantly between the acnegenic and the normal skin subjects or between photodamaged/aged and normal skin subjects, indicating that skin type did not influence the rate or extent of SA absorption. Previous studies with abraded animal skin and diseased human skin demonstrated that SA absorption was significantly greater through compromised skin, regardless of skin delivery vehicle.^{2,12,20} For example, in a study by Taylor and Halprin,⁴ the average topical absorption from a 6% SA cream was ~70-80% of the applied dose in psoriatic patients. Relative to those reports,

Table 3—Steady-State SA Pharmacokinetic Parameters in Subjects Presenting Normal, Aged or Acneogenic Facial Skin after Topical Application of 2% SA or in Subjects Receiving One Daily Oral Dose of 81 mg ASA^a

Skin Type	Vehicle	Peak Plasma Salicylate Levels (μg/L)	Time to Peak Plasma Salicylate Levels (h)	Terminal Exponential Half-Life Plasma Salicylate (h)	Area under the Curve Plasma Salicylate Levels (μg h/L)
Normal	Cream	293 ± 37	4.30 ± 0.40	5.83 ± 0.73	3108 ± 293
Aged	Cream	275 ± 58	4.11 ± 0.58	5.93 ± 0.83	2836 ± 302
Normal	Hydroalcohol	525 ± 66 ^b	1.89 ± 0.35 ^b	7.62 ± 0.82	4225 ± 425 ^b
Acneogenic	Hydroalcohol	487 ± 41	1.67 ± 0.24	8.08 ± 1.12	3893 ± 329
N/A	Oral ASA ^c	5282 ± 457 ^d	0.71 ± 0.25 ^d	2.62 ± 0.46 ^d	22010 ± 3907 ^d

^a Data presented are the mean ± SEM for $n = 10$ (normal/cream group) or $n = 9$ (all other groups). ^b Significantly different from subjects presenting normal facial skin treated with 2% SA in a cream vehicle ($p < 0.05$). Significant differences were not found within vehicle type in subjects presenting normal compared with aged or acneogenic facial skin. ^c N/A, Not applicable. Subjects scheduled for oral ASA treatment were not required to meet facial skin criteria. ^d Statistically different from all topical treatments.

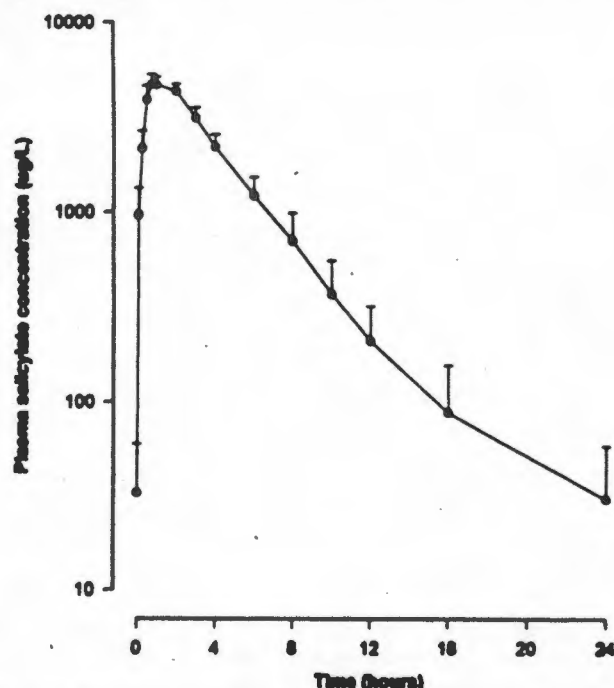


Figure 2—Plasma concentration-time profile of SA in subjects receiving oral administration of 81 mg of ASA. The data presented are the mean ± SEM for $n = 9$ subjects at each time point. (Note the 10-fold change in the y axis compared with Figure 1.)

Table 4—24-Hour Cumulative Urinary SA Excretion in Subjects Treated Topically with 2% SA or Orally with 81 mg of ASA^a

Skin Type	Vehicle	Total 24-Hour Cumulative Urinary Salicylate Excretion (mg)
Normal	Cream	6.10 ± 1.01
Aged	Cream	6.44 ± 0.94
Normal	Hydroalcohol	5.58 ± 0.69
Acneogenic	Hydroalcohol	6.00 ± 0.67
N/A ^b	Oral ASA	37.58 ± 4.02 ^c

^a Data presented are the mean ± SEM for $n = 10$ (normal/cream group) or $n = 9$ (all other groups). ^b N/A, Not applicable. Subjects scheduled for oral aspirin treatment were not required to meet facial skin criteria. ^c Statistically different from all topical treatments.

the present data indicate that neither of the skin types we evaluated constitute a compromised barrier for SA absorption.

In the present study, bioavailabilities for topically applied SA among normal skin type subjects were 57.6 and 44.0% for the hydroalcoholic and cream delivery vehicles, respectively. The lower absorption of topically compared with orally administered salicylates observed in this study is in agreement with earlier reports by other investigators.^{24,25} More-

over, the slower half-life observed after topical compared with oral administration indicated that absorption is the rate-limiting step for absorption of topically applied SA.

This study has provided new data on the percutaneous absorption of SA from hydroalcoholic and cream vehicles and on the impact of different skin types on bioavailability of the compound. This pharmacokinetic information allows for improved estimates of systemic SA contributions from topically applied products. Based upon the outcome of this study, systemic exposures to SA from the use of topical 2% SA products are ~15% of those obtained following oral administration of 81 mg of ASA and substantially below those associated with adverse SA-related effects.

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ENCEPHALOPATHY AND FATTY DEGENERATION OF THE VISCERA A DISEASE ENTITY IN CHILDHOOD

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THIS report describes the clinical and pathological features of a group of twenty-one children admitted to the Royal Alexandra Hospital for Children between March, 1951, and March, 1962, who appear to have had an illness which, we believe, represents a clinicopathological entity. We shall refer to this condition as fatty degeneration of the viscera, of unknown cause.

The outstanding clinical features were profoundly disturbed consciousness, fever, convulsions, vomiting, disturbed respiratory rhythm, altered muscle-tone, and altered reflexes. The onset was usually associated with cough, rhinorrhoea, sore throat, or earache. There was often hypoglycaemia and a low cerebrospinal fluid (C.S.F.) glucose, and the serum glutamic-oxalacetic acid transaminase (S.G.O.T.) and serum glutamic-pyruvic acid transaminase (S.G.P.T.) levels were increased in each of the seven patients in whom they were measured.

Seventeen of the children died, and at necropsy remarkably uniform pathological changes were found. To the unaided eye, these were expressed as cerebral swelling, a slightly enlarged, firm, and uniformly bright-yellow liver, and pallor and slight widening of the renal cortex.

Clinical Features

The series consisted of fourteen girls and seven boys. The youngest was 5 months old, and the eldest 8½ years. Fourteen of the twenty-one were aged 2 years or less, and the other 7 were over 5 years.

The frequency of the main clinical features and the laboratory findings is shown in the tables.‡ There was usually an initial period of malaise, often with cough, sore throat, earache, or rhinorrhoea, and the children did not seem very ill. Usually after 1 to 3 days, but varying occasionally to 2 or 3 weeks, there was fairly abrupt clinical deterioration, the most worrying changes being

persistent severe vomiting and the onset of stupor or coma, sometimes followed by convulsions. In ten of the fourteen children who had upper-respiratory tract symptoms, there was a short period of apparent recovery before symptoms returned and the more serious phase of the illness supervened.

In nearly half the group there was wild delirium, with screaming, intense irritability, and violent movements, during the period when consciousness was deteriorating, and in several of the other children an unusual degree of restlessness was noted.

On admission to hospital, all but two of the patients were already stuporose or comatose. Eight exhibited, at some time afterwards, the delirium described above. Usually one or more of the features that we have learnt to regard as characteristic, such as hyperpnoea or a low C.S.F. glucose, was present at or shortly after admission, and others appeared over the ensuing 12 or 24 hours.

Every patient had profoundly disturbed consciousness, and seventeen had seizures. The types of convulsion varied, and some patients had more than one sort of fit at different times. Focal twitching and tetanic spasms were seen most often, and in three patients, one of whom had opisthotonus, the spasms were sufficiently like tetanus for this diagnosis to be considered. Convulsions were often difficult to control; in ten of the seventeen patients who had fits these lasted more than 3 hours, and in four they were terminated only by death.

Vomiting was another constant symptom, and in most patients it was severe. Eleven patients vomited black or dark-brown material. It was notable that vomiting invariably ceased or became much less frequent shortly after admission, that is, a few hours after the onset of stupor or coma.

The respiratory rhythm was noted to be abnormal in twenty cases, and most exhibited changing patterns of breathing. Hyperpnoea and irregular respirations were the commonest abnormalities noted, but shallow or rapid breathing also occurred, and one patient, who became apnoeic, remained alive for 26 hours with assisted respiration.

The liver was palpable in twelve, and in seven it seemed, on repeated examination, to have become larger than on admission. In six the liver was thought to be abnormally firm, and this was, in our experience, a more helpful finding than apparent enlargement.

Muscle-tone and tendon-reflexes tended to be changeable. Increased or variable tone and tendon-reflexes were found in sixteen. The plantar responses were abnormal or equivocal in about half of the group, and they were often variable from time to time in the same patient. The pupils became dilated or unresponsive to light, or both, in ten patients. In only one patient were the pupils unequal.

A characteristic posture was observed—the elbows flexed, the legs extended, and the hands clenched—and some or all of these features were noted in seven patients. This finding, in common with hyperpnoea, firmness or enlargement of the liver, and changes in the muscle-tone and tendon-reflexes, was more often recorded in the more recently admitted cases, when clinicians at the hospital had become familiar with the condition.

In twelve patients ketones were found on urine analysis, or there was a ketonic odour on the breath. Every patient except one had fever while in hospital, but this was not

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‡ These may be had from *The Lancet*, 7, Adam Street, Adelphi, London, W.C.2.

usually present on admission. Five patients appeared to have abdominal pain at some stage of the illness. In each case this was brief.

Four patients had a rash. In three this was a non-specific erythematous eruption of short duration, but in the fourth patient it was an unusual and striking feature of the illness. The rash had started on the front of the neck and had become generalised. The lesions were discrete pink papules which became excoriated and crusted. On admission after the rash had appeared, a diagnosis of varicella with encephalitis was made. The patient died, and at necropsy the typical changes of fatty degeneration of the viscera were found. The skin lesions were histologically quite unlike those of varicella, being strictly circumscribed foci showing necrosis of the epidermis and the full thickness of the dermis, with some leucocytic infiltration and the formation of micro-abscesses at the edges of the lesions.

Seventeen of the twenty-one patients died, the average time of survival after admission being 27 hours, and the longest survival $2\frac{1}{2}$ days. Vomiting invariably subsided shortly after admission, but the disturbance of consciousness did not improve, and, together with disturbed respiratory rhythm, convulsions, or peripheral circulatory failure, or a combination of these, this dominated the clinical picture until death.

Four patients survived the illness. Each of them was given intravenous glucose by infusion and two received hydrocortisone intravenously as well, one was given insulin and glucose, and one was given both hydrocortisone and insulin. In each case consciousness was normal by about 24 hours after improvement was first detected. Within a day or so of regaining consciousness each of the patients had returned to apparently normal health and remained well thereafter.

Laboratory Investigations

The following are the investigations which often gave abnormal results (see also table III):

The *C.S.F.-sugar concentration* was estimated either qualitatively or quantitatively. The qualitative test was performed by boiling equal quantities of *C.S.F.* and Benedict's solution. Where no reduction occurred, this is reported as "negative", and the result is considered to represent a value for *C.S.F.* sugar of less than 25 mg. per 100 ml. The *C.S.F.-sugar concentration* was estimated before intravenous glucose was administered, in nineteen patients. In thirteen of these the concentration was reduced, although in two this was only detected in a second specimen of *C.S.F.*, the first being normal.

The *blood-sugar level* was estimated before glucose was administered in nine patients. It was less than 50 mg. per 100 ml. in six, 62 mg. per 100 ml. in one, 195 mg. per 100 ml. in another, and normal on 2 occasions in one other. In the patient whose blood-sugar level was 62 mg. per 100 ml., the *C.S.F.* had given a negative qualitative test for sugar.

The *blood-urea* exceeded 40 mg. per 100 ml. in nine of the twelve patients in whom it was measured. Five of these were clinically dehydrated, and two were considered not to be dehydrated. In one patient the blood-urea was 64 mg. per 100 ml. on admission, and it rose to 180 mg. per 100 ml. next day, although signs of dehydration had not worsened and they were never of more than mild to moderate severity.

The *S.G.O.T.* was estimated in seven patients, and it was raised in each case. Initial levels varied between 106 and 170 Karmen units per ml. per minute.

The *S.G.P.T.* was estimated in four patients, and it was initially raised in all to between 155 and 1200 Karmen units per ml. per minute.

The *prothrombin-index* was estimated in four, and it was low in each of these.

Neutrophil leucocytosis was commonly observed.

Electroencephalography was performed in six cases, and gross generalised abnormalities were found in each. Repeated tracings were made in two patients who survived, and a return to normal was observed.

Chromatography of the urinary aminoacids was performed in six instances. The pattern in one case was normal, and the other five showed a generalised increase in the concentration of aminoacids.

Blood-cultures were performed twice, and there was no growth of organisms in either case.

Clinical Diagnosis

In a number of patients admitted with stupor or coma, with or without fits, and with a history of vomiting, it has been possible to make an antemortem diagnosis of fatty degeneration of the viscera. The most helpful diagnostic findings in such patients have been hypoglycaemia, with no immediate response to intravenous glucose, low *C.S.F.* sugar, hyperpnoea, increased or variable muscle-tone and reflexes, firmness of the liver, and raised serum-transaminase levels. A history of unusually severe vomiting, progressing to a stage in which black vomitus has run effortlessly from the mouth of the stuporose patient, has sometimes suggested the diagnosis.

Pathological Features

Irrespective of the period of survival from the time of onset of serious symptoms, the gross and microscopic pathological changes found in the seventeen cases which came to necropsy was, apart from some minor variations, identical from case to case.

Gross Changes

The brain was always swollen, and this swelling was sufficient to produce obvious flattening of the cerebral convolutions, but it never reached a degree sufficient to produce herniation of tissue, and only rarely was any flattening of the pons and medulla or coning of the cerebellum apparent. The cut surfaces were pale and moist. The liver was slightly enlarged, and it felt unduly firm, and the capsular and cut surfaces were uniformly bright yellow. The kidneys had a slightly widened cortex which was pale, with a faint yellow tinge. The upper-respiratory tract was not inflamed, and there were no pulmonary pathological changes apart from a little oedema in some cases. The heart was usually dilated, and in a few instances the myocardium, when viewed from the endocardial surface, had a faint yellow tinge. In the majority of cases the stomach contained a small quantity of dark fluid coloured by altered blood, and in a few there were multiple superficial erosions.

Microscopy Appearances

The cerebral changes, which were in no sense specific, were principally located in the cerebral cortex, but they were not restricted to any specific area nor to any one lamina of cortical cells. Cortical neurones showed one of two changes: in one form the cell was considerably swollen; in the other the cell was shrunken and deeply staining. Cells of both types were intimately mingled, though in some areas several microscopic fields were composed almost exclusively of one or the other type. The striking feature of the enlarged cells was the pallor of the cell body which, in the paraffin sections, appeared empty or contained a few coarse refractive granules; in frozen sections a few cells of this type were found to contain a number of sudanophilic granules. Fat droplets were also seen in a few of the capillary endothelial cells in these sections, but fat emboli were not found in the vessels. In the deeply staining cells the cell body and the nucleus were contracted; the cytoplasm was opaque, and the nucleus was dense with a hazy outline and no visible nucleolus. In occasional cases eosinophilic necrosis of

the cortical neuro- distribution—a pattern in patients who die

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In the tissues of fatty degeneration cases and the myo limited extent in a two latter organs, paraffin sections su so minor degrees of more often than the

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the cortical neurones was found, sometimes in a laminar distribution—a pathological change which is often discovered in patients who die in convulsive states from many causes.

In all regions of the brain the astrocytes and oligodendrocytes were greatly swollen, though the cell bodies did not contain fatty droplets. There was no proliferation of the microglia and no evidence of any inflammatory reaction in the meninges or cerebral tissue, and the myelin was well preserved in all regions.

In the tissues other than neural, the changes were those of fatty degeneration which affected the liver and kidneys in all cases and the myocardium and pancreatic acinar cells to a limited extent in a few. Frozen sections were made of these two latter organs, however, only when the examination of paraffin sections suggested the possibility of fatty change, and so minor degrees of fat accumulation could have been present more often than the record suggests.

In the liver it is the uniformity and completeness of the fatty change that is such a striking feature; every cell in every lobule is packed with fatty droplets. In addition, many cells contain a few optically empty vacuoles, even where the frozen sections were made from material embedded in gelatin, suggesting that these empty spaces may represent unstainable material rather than vacuoles from which the fat had been dislodged during processing. Despite this extensive fatty change, there was no necrosis either of zonal distribution or of individual cells, nor were mitotic figures or binucleate liver cells present as indirect evidence of prior cellular dissolution.

There were two cases which, though resembling the group in all other aspects, did show some variation in the liver. In both the liver felt a little softer than normal rather than unduly firm, and in one case it was slightly reduced in size. In addition to the uniform fatty change both these cases showed a narrow zone about the portal tracts of cellular necrosis, and in one (the case in which the liver size was a little reduced) there was an accumulation of inflammatory cells, which were principally lymphocytes and plasma-cells, but with occasional polymorphs in the portal tracts.

In the majority of cases portions of liver were placed in an alcoholic fixative during necropsy and stained for glycogen. Very little glycogen, and certainly no glycogen in excess, was found in any of these sections. Because of the uncertainty which still remains regarding the estimation of glycogen in necropsy material, however, this aspect will be mentioned again when the biopsy specimens are discussed.

In the kidney fatty degeneration was obvious in the proximal convoluted tubules and in the loops of Henle; occasional droplets of fat were present in only a few cells of the distal tubules. The glomeruli, vessels, and interstitial tissues were entirely normal. Apart from the fatty change found in the myocardium and pancreas (already remarked upon), histological study of the other organs, including the endocrine glands, revealed no pathological changes.

In the specimens from liver biopsies performed during the active stage of the illness in two patients, the changes in the liver cells were identical with those seen at necropsy in all cases. The biopsy specimens differed from the necropsy material only in that glycogen could be demonstrated in the cells in amounts considered to be within the normal range. In one case the S.G.O.T. level had fallen from 1200 to 530 Karmen units at the time of the first biopsy. The biopsy was repeated in this patient 3 weeks later, when the S.G.O.T. level had fallen to 37 Karmen units and the patient was clinically well. In this second biopsy specimen the liver tissue was histologically normal in every respect.

Treatment

It cannot be proved that any form of treatment has altered the outcome. We have gained an impression, however, that the continuous infusion of glucose and the concomitant use of a corticosteroid improved the chances of survival. It was a feature of the illness that a single dose of glucose by intravenous injection did not bring about the expected improvement in the hypoglycaemic

patients. When glucose was given by infusion the results bore some relation to the rate of administration and to whether hydrocortisone was given as well. Five children received glucose by infusion at a rate less than 2.5 g. hourly, and no improvement was seen. Among six who were given larger doses of glucose, without corticosteroids, there was one survivor. Seven children were given glucose infusions at rates greater than 2.5 g. hourly, together with hydrocortisone in high dosage, and of these three survived. Since we had become accustomed to regarding the outlook as almost hopeless, this has created a fairly strong impression that the treatment had some bearing on the outcome.

Insulin was sometimes given with the idea of enhancing the utilisation of the infused glucose. Two of the four children who survived received soluble insulin.

Discussion

When the first patient of the series was admitted to hospital in 1951, he was thought to be suffering from encephalitis or septicæmia. When a full necropsy study had been completed it was evident that neither of these diagnoses was likely. However, the possibility of an unusual degree of neuronal swelling and fatty degeneration of the liver and kidneys as the result of septicæmia or even viræmia by an unidentified organism was not considered to be disproved. While accepting the non-specific character of the pathological changes when viewed separately and out of context with the history, the impression remained that this was an unusual case, that the pathological findings were unexplained, and that no exactly similar case had come to necropsy in this hospital in the preceding 10 years.

Some 2 years elapsed before the second patient presented, and from this case and the next organs were submitted for toxicological examination. It was not possible to guide the toxicologist in his investigations further than to suggest a routine study for the more commonly encountered poisons, especially those likely to produce fatty degeneration without cellular necrosis as the only definable microscopic pathological change. These studies were unfruitful; but no exhaustive toxicological analysis has ever been undertaken.

As further patients presented with similar clinical features, and the pathological pattern remained constant regardless of the duration of the illness preceding death, we became increasingly convinced that we were dealing with a clinicopathological entity, and one sufficiently distinctive to allow a presumptive clinical diagnosis to be made sometimes. We are not, of necessity, entirely convinced that the ætiology is identical in every case.

When seven patients had been seen, a retrospective investigation was made of the illness in the five most recent cases, through the cooperation of the Institute of Child Health in Sydney. A medical officer of the Institute visited the homes of the children concerned. His investigation included a history-taking of any illness in the family at about the time the patient became ill, a general review of the patient's immediate environment, and a searching inquiry as to possible access to drugs and poisons.

More recently a further study of this type was conducted by the department of public health in the State of New South Wales. The visits and interviews were more closely related to the patient's illness. In addition to the more general inquiries made in the first survey special attention was paid to the possible ingestion or inhalation of carbon tetrachloride or trichlorethylene, and in view of

the hyperpyrexia in some patients, of dinitrate cresol or dinitrate orthocresol (the pesticides used against red-spider infestations). This survey, like the first, failed to provide evidence of access to any likely poison or to show any relation to concurrent illness in parents or siblings.

Few conditions resemble the fatty-degeneration syndrome at all closely. There is a strong similarity to the case described by Curry et al. (1962) in an 8-year-old boy, who died after being ill for 6 days. His illness began with vomiting, abdominal pain, and screaming fits, and then passed into stupor. When admitted to hospital 6 hours before he died, he had a C.S.F.-glucose level of 15 mg. per 100 ml. and a blood-sugar level of 75 mg. per 100 ml. No other investigations were contributory, and serum-enzyme studies were not recorded. At necropsy his body was slightly jaundiced, the liver was intensely yellow, and severe fatty change was found in the liver microscopically. Fatty change was also found in the heart-muscle and kidneys. Thorough toxicological examination failed to reveal any toxic substances. This case-report is of special interest because the urine contained an abnormal substance which proved to be a pteridine. Whether this substance was also responsible for the bright-yellow colour of the liver seen in our series has not been investigated.

The vomiting sickness of Jamaica bears certain resemblances to this fatty-degeneration syndrome. This illness is peculiar to Jamaica, and there is a diminishing incidence after the age of 10. The onset is sudden, with violent vomiting followed by drowsiness and coma, and the mortality-rate is high though variable (Hill 1952).

Fatty change of the liver, kidneys, and other organs is a prominent feature, but the widespread oedema of connective tissues, swelling and hyperaemia of the lymph-nodes, and necrosis of liver, kidney, and pancreatic cells are all features which do not appear in the necropsy material in our series, and we have been unable to reveal a likely source for a vegetable poison such as that believed to cause the vomiting sickness of Jamaica.

Cases similar to those recorded here have occurred in other Australian States. To our knowledge these patients have not been reported as a group, although some of the children with acute encephalopathy described by Anderson (1963), seem to have had illnesses very like the one we have described. Anderson attributed the changes outside the nervous system to secondary nutritional effects in a primarily neurological disorder, rather than an integral part of the overall disease process.

We have recorded the details of this series because we are convinced that they form a group different from those children in whom fatty changes, especially in the liver, are a secondary manifestation of a variety of diseases. We hope that the experience of others may help to suggest an answer to the problems of aetiology, prevention, and treatment.

Summary

The clinical and pathological features have been described in twenty-one children with encephalopathy and fatty degeneration of the liver, kidneys, and sometimes other organs.

It is suggested that the illness of these children represents a clinicopathological entity of unknown aetiology.

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BONE RESORPTION AND OSTEOPOROSIS

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BONE is a living tissue which is constantly being laid down and resorbed throughout life—more rapidly in the young than in the elderly. When the resorption outstrips the laying down of new bone, osteoporosis develops. This word "osteoporosis" simply means that there is less bone than one would normally expect; it is not a description of a specific disorder. From the age of about 30 onwards the amount of bone normally decreases.

The first difficulty has always been to decide where the difference lies between normal and abnormal bone resorption, and the second to decide whether there is, in fact, any such definite border, even though gross resorption is clearly abnormal.

In an attempt to clarify the picture, this subject of osteoporosis will be considered within the whole context of bone deposition and removal. Looked at in this way it can be shown that the various types of osteoporosis manifest themselves as a consequence of a certain type of cell behaviour. When many cells display this activity there is clear-cut osteoporosis; when few cells are active there is borderline osteoporosis which may produce no clinical manifestations. Here two types of osteoporosis will be described: localised "disuse osteoporosis"; and the type whose cause is as yet unknown, and which commonly leads to fractures in the elderly—more particularly in elderly women. Clinically this is the most important type, because of the large number of people affected.

Bone Deposition and Removal

The mechanism of bone formation is quite different from that of calcification. From many careful observations recorded in the second half of the 19th century and the early years of the present century it became known that the necessary conditions for ossification were the presence of calcified tissue (formed normally or abnormally) and the penetration of blood-vessels. Thomas²⁹ had shown that calcareous foci were gradually eroded by proliferating blood-capillaries, and that as a result of an unknown stimulus (believed to be the presence of the calcium salts) some of the mesothelial cells of the new blood-vessels assumed the role of osteoblasts, and thus originated bone. In his description of a bone—complete with marrow cavity—formed within an artery wall, Bunting³⁰ stated that lamellar bone "contains numerous typical bone corpuscles, and is penetrated at its thickest portion by a capillary vessel surrounded by a layer of cells resembling osteoblasts closely applied to the wall of bone, the whole appearing to represent a Haversian canal".

During the past ten years Trueta and his co-workers^{31, 32, 33, 34, 35} have carefully re-examined the mechanism, making use of additional techniques now available. They have provided convincing evidence that the osteoblasts which lay down bone are members of a series of related cells derived from the cells of the sinusoid vessel walls (vessel-wall cell—intermediate cell—osteoblast—osteocyte) and joined together as a syncytium—thus, all the cells in the group are joined to one another through their cell processes (figs. 1–3). In developing bone, the matrix produced by osteoblasts is laid down on calcified cartilage; thereafter it is laid down on previously existing bone.

Normal resorption takes place along the surfaces of the bone, and osteoclast formation is frequently observed.



Fig. 1.

Fig. 1—Syncytium, (right). Intercellular plane of section. (X 1000.)

All illustrations are by the author.

Fig. 2—Cell in syncytium and surface of calcified tissue.

Trueta and Rigal³⁶ have shown that a syncytium coalesces to form a single cell. When osteocytes are resorbed they are preceded by the resorption of the bone between the osteoclasts and the osteoblasts and into the syncytium, coalescing to form a single cell. These changes are consistent with the chemical that could be the bone tissue. The apparently of the bone integrate and are resorbed.

This, very briefly, is the process of bone resorption. The mechanism, whether in the type of bone being considered, is a process of resorption. There are a number of labelling which show these two types.

Characteristics

In observation of osteoporotic tissue, the of badly affected tissue show differences to be justified, because tissues have a pathological cases, where there are differences to be applied working on normal tissue.

Disuse osteoporosis in laboratory animals³⁶; human



Fig. 3.

Fig. 3—Cortical bone sulphate, used as a marker (by half.)

Cell, on vessel wall, processes passing through processes.

Fig. 4—Normal bone. Osteoclast on left surface is partly demineralised.

A Study of Sixteen Fatal Cases of Encephalitis-like Disease In North Carolina Children

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The etiology of diseases and deaths reported as encephalitis represents one of the most perplexing problems in the field of infectious disease—to the clinician, the epidemiologist, and the virologist. In 1960 this ill-defined entity, "infectious encephalitis of unknown etiology," accounted for 1335 or 52 per cent of all cases of infectious encephalitis reported to the Encephalitis Surveillance Unit, Communicable Disease Center, Atlanta, Georgia¹. In 1961 encephalitis of unknown etiology was by far the largest category (56.3 per cent of a total of 2143 cases reported by state epidemiologists). Mumps and measles encephalitis represented 31.5 per cent and arthropod-borne, varicella, influenza, post-vaccinal, and unspecified encephalitis represented the balance.

Early in 1962 a rapid increase in the reporting of fatal encephalitis-like disease in North Carolina children was noted. From January 1, 1962, until April 30, 1962, a total of 27 cases involving children 15 years of age and under was reported to the North Carolina State Board of Health. Preliminary evaluation of the cases reduced the number of cases of true encephalitis-like disease in children to 16. The other 11 cases were due to causes rather conclusively not encephalitis, or causes without enough documental information for proper evaluation. A survey of all reported deaths of meningoencephalitic disease of unknown etiology in this age group in the previous five years in North Carolina demonstrated this increase.

The numbers cited in table 1 represent the total number of cases of meningoencephalitis of unknown etiology by year. It

appeared to us that an unprecedented phenomenon, as evidenced by the number of encephalitis-like deaths in this age group, was occurring in North Carolina.

Table 1
Cases of Fatal Meningoencephalitic Disease of Unknown Etiology*

Year	No. Cases
1957	9
1958	15
1959	11
1960	5
1961	9

*International Classification of Diseases. 340.2 meningitis, except meningococcal and tuberculous, due to other specified organism; 340.3 with no specified cause; 343 encephalitis, myelitis, and encephalomyelitis.

We were further stimulated by a letter from the Department of Health, Commonwealth of Pennsylvania², which reported four deaths in children aged 7½, 8, and 10 years, respectively, in January, 1962. These children had a relatively mild upper respiratory disease which was followed by a period of apparent recovery. Three to five days later, however, there was an onset of fever, vomiting, and hyperirritability which progressed to produce drowsiness and coma. In spite of supportive therapy, the course of each patient was described as rapidly downhill, with death occurring approximately 48 hours after the onset of vomiting. These cases seemed remarkably similar to those occurring in North Carolina.

Investigation

We began to investigate each case as rapidly as possible after it was reported. An attempt was made to accumulate all pertinent facts relating to the history, physical and laboratory findings, postmortem examination, and bacteriologic and virologic materials. Inquiries were made of attending and referring physicians, pathologists, hos-

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pitals, and the families of the children, and all the information was carefully appraised.

The 16 cases were broadly distributed geographically, as demonstrated in figure 1. This figure also shows the distribution of cases by month of onset. All but three of the children came from predominantly rural areas. Figure 2 demonstrates the distribution of cases by month of onset and race, sex, and age. It is of particular interest that the peak of the cases reported coincides with that of reported influenza B in North Carolina during 1962.

Case Summaries

Accompanying charts represent all positive information available from each of the 16 cases. Since in many instances there was a delay between the actual death and our notification it was impossible for us to obtain the necessary specimens and studies in every case—which would have been ideal.

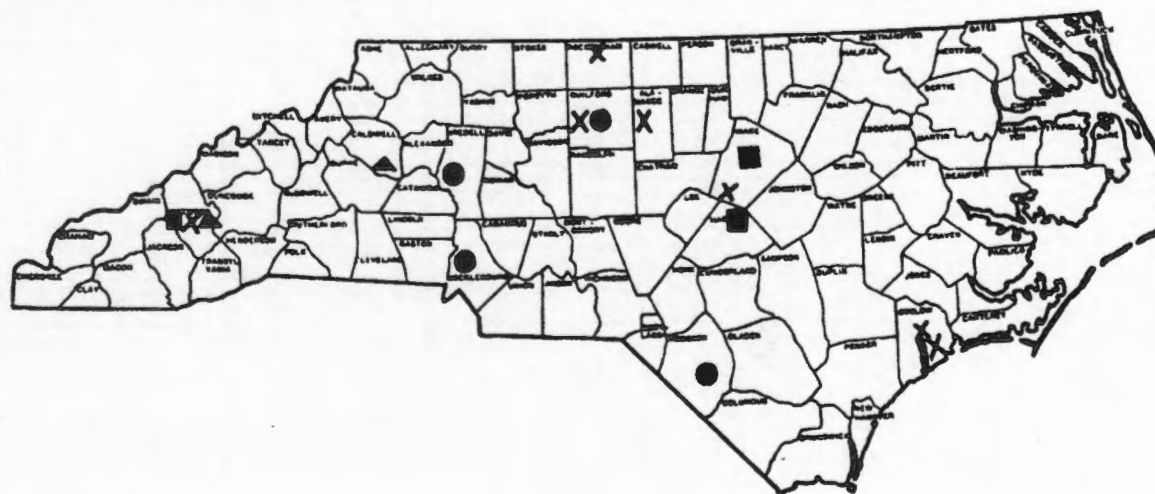
Signs and symptoms

Premonitory signs and symptoms were generally similar and almost uniformly mild. In 4 of the cases the onset of the en-

cephalitic syndrome appeared within less than 24 hours after the onset of illness. In 2 other children, premonitory signs and symptoms were less than three days in duration. Similar to the Pennsylvania report, 4 of the patients manifested mild, nonspecific signs and symptoms for three to five days before the onset of severe illness. The remaining 5 had symptoms from five to eight days before the onset of apparent encephalitis. Only 1 of the 16, an 8 year old juvenile diabetic, had a significant past medical history.

As noted on the case summaries, 2 children complained mainly of leg pain; emesis was prominent in 6, sore throat in 2, lethargy in 2, and cough in 3. Six of the 13 experienced mild respiratory disease that occasioned no particular parental concern. Low-grade fever was noted in only 4 children prior to the beginning of the devastating symptomatology.

At the onset of the encephalitis-like illness, marked elevations of temperature were recorded in 6 children; and tonic-clonic movements, convulsions, or both, were present in 10 of the 16 children. Eleven



- X - JAN., 1962
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Fig. 1. Geographic location and month of onset.

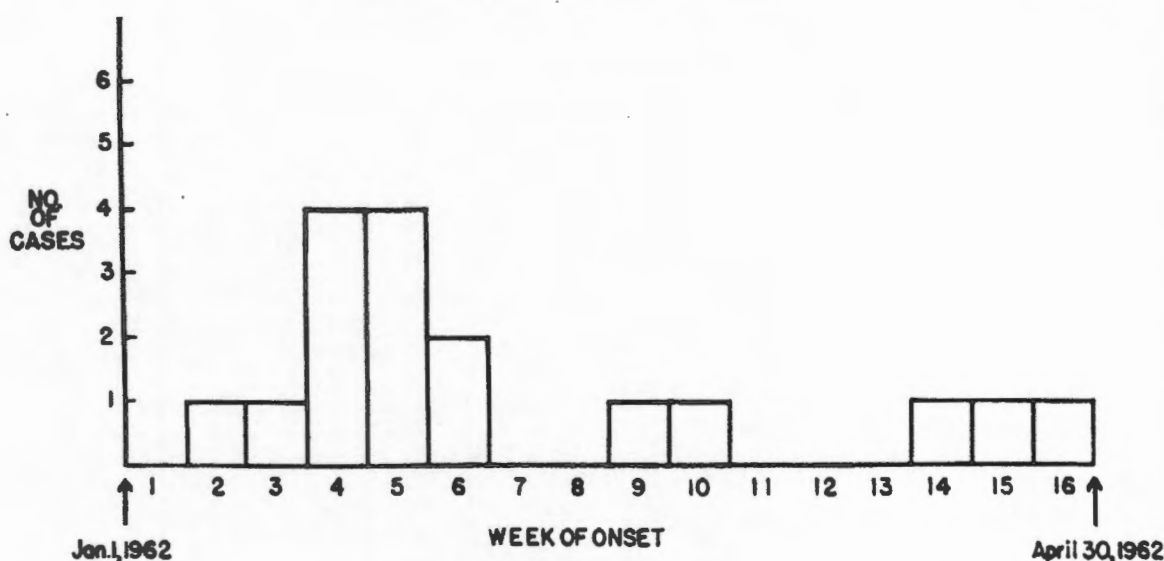


Fig. 2. Cases by week of onset.

Table 2
Age Incidence

Years	No.
0-1	2
1-3	3
6-10	7
6+	3

Table 3
Race and Sex Incidence

White	15
Non-White	1
Male	6
Female	10

patients had respiratory depression on admission. All 16 children rapidly lapsed into coma and expired.

Laboratory findings

Spinal fluid findings in the 16 cases showed considerable variation. In most instances, opening and closing pressure readings were not available. In only 2 instances was the spinal fluid pressure reported as elevated, and in only 2 others did the spinal fluid reveal more than 6 blood cells. The spinal fluid protein was elevated in 4 cases, 2 of which had elevated cell counts. Spinal fluid sugar was reported above normal in 3 instances.

White cell counts showed much the same kind of variation. Seven specimens demonstrated more than 20,000 per cubic millimeter, and only 4 had less than 10,000. In general, the differential counts were not remarkable. Considering the severity of the

cases, the blood chemistry values were not remarkable. The few abnormal blood chemistry studies reported were consistent with the particular cases.

Autopsy findings

Fortunately, postmortem examinations were done immediately following 13 of the 16 deaths. Marked cerebral edema seemed to be the only consistent finding, being observed in 7 of the 10 cases. There was marked fatty metamorphosis of the liver in 4 cases. In 2 cases microscopic cardiac changes in the form of myocardial necrosis and fatty degeneration of the myocardium were reported. In one case fatty metamorphosis of the liver and associated fatty degeneration of the myocardium and kidneys were demonstrated.

It is interesting to note that in 2 cases the fatty metamorphosis of the liver was similar to that seen in acute phosphorous intoxication. Toxicologic studies of tissue failed to confirm the latter. In one child (case 11) autopsy demonstrated central nervous system findings suggestive of infectious encephalitis (hemorrhagic and lymphocytic perivascular infiltration in the brain). This patient had associated spinal fluid changes. Since this patient also had an elevated spinal fluid protein (61 mg. per 100 ml.) and an increased spinal fluid cell count, (56 cells with 88 per cent lymphocytes), it is the only case in the series which

SUDDEN DEATHS IN CHILDREN

NO.	AGE	RACE	SEX	CLINICAL SIGNS AND SYMPTOMS	LABORATORY FINDINGS	POST-MORTEM FINDINGS	VIRUS FINDINGS	
							Specimens Available	Results
1.	N.Y. 7	W	F	Few aches and pains in back of legs for two days. Sudden onset of Jacksonian type convulsion. Afebrile on admission. Fever to 100° developed shortly. Decorticate rigidity developed and 20 hours after admission another seizure occurred followed shortly by respiratory arrest.	WBC - 20,260 with 70% segs CSF - Normal, protein - 45.5mg.% BUN - 8.25mg.%; Blood sugar - 23mg.% Blood culture - no growth	Cerebral edema both grossly and microscopically. No evidence of septic meningitis. There was damage to brain mainly in area of pons, characterized histologically by vacuolization of the myelin and loss of staining quality. Marked pulmonary edema and intra-alveolar hemorrhage.	Serum specimens obtained three days apart. Brain	ECHO 9 isolated from brain. The pt's sera did not neutralize virus isolated from brain.
2.	S.V. 9	W	M	Vomiting for three days. Admitted to hospital dehydrated. Shortly after admission had a generalized convulsion. Followed by thrashing about in bed and intermittent tonic-clonic seizures. Afebrile on adm. Comp ensued. Temp. rose to 100° and respirations became labored. Pt. expired 28 hours after admission.	WBC - 9200 with 70% segs CSF - Protein and sugar normal, pressure - 120 mm.	No autopsy.		
3.	N.H. 18	W	F	History of lobar, headache, mild gastric pain, vomiting and excruciating; 4 days pta she began vomiting. One day pta - disoriented with purposeless movements. Tracheotomy on admission failed to halt respiratory distress. She was maintained on a respirator and weaned for the next few days, until death.	WBC - 17,360 with 85% polys CSF - Protein - 124 Blood sugar - 100mg%; BUN - 37mg% Ca-103, K-57, Cl-106, CO ₂ -20. mg/l Cold agglutinins-1:40 CSF culture, rectal swab, urine culture - neg. ENG-paroxysmal tachycardia with variable block EEG-profoundly abnormal with disturbance of cortical rhythm over both hemispheres.	Brain showed wide-spread neuronal necrosis, early degeneration of myelin, but no inflammatory reaction in either the brain or meninges. Pituitary shows infarction of anterior lobe. Heart-spotty myo-cardial necrosis. Liver-marked fatty metamorphosis.	Blood, spinal fluid, stool, throat swab.	Negative
4.	S.N. 8	W	F	Intermittent upper respiratory symptoms for eight days pta. Lethargy began 2-3 days pta. Vomiting frequently during the 24 hours pta. On admission child restless and confused. Became increasingly drowsy, reacting only to deep, painful stimuli. Despite digitalization and endotracheal suction, the child died two days after admission.	WBC - 47,300 with 35% neutrophils CSF - negative CO ₂ -19.4; K-5.5; Cl-111; Na-150 mg/l; BUN-18.2mg% Transaminase-392 SGT units; Total bilirubin-0.8mg%	Marked cerebral edema with herniation of cerebellar tonsils through the foramen magnum, focal hemorrhages--cerebral cortex, broncho-pneumonia, pulmonary edema, vascular congestion, marked fatty change of the liver.	None	

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NO.	AGE	RACE	SEX	CLINICAL SIGNS AND SYMPTOMS	LABORATORY FINDINGS	POST-MORTEM FINDINGS	VIROLOGY FINDINGS	
							Specimens Available	Results
6. W.S.	12	W	F	Two days pta felt tired and listless. Intermittent vomiting began. Shortly before admission became completely uncoordinated, inattentive and unresponsive, going into coma with carpo-pedal spasm, muscular tremor, and tightness of extremities. Temperature 102° on admission. Pupils fixed and dilated, not responsive to pain, child rapidly became more deeply comatose with respiratory depression and expired 13 hours after admission.	WBC - 10,900 with 61% segs CSF - 6 cells, (2 segs, 4 small mones), Protein-32.5 mgm% Sugar-93 mgm% Na-140; CO ₂ -18.6; Cl-112 meq/l; BUN-42.5 mgm%; Transaminase - 1270 SGT units Lung culture-positive Staph Albus Spinal fluid culture-no growth Blood culture - no growth Liver - negative for phosphorus, heavy metals, lead	Edema of the brain. Small focal hemorrhages in the brain. Meningo-encephalitis and meningo-eyelitis (small amount inflammation in a few small foci). Intra-pulmonary hemorrhage and acute bronchitis. Fatty degeneration of the myocardium. Marked fatty metamorphosis of the liver. Fatty degeneration of kidney.	Liver, lung, heart, feces, brain from patient. Stool specimens and sera from parents.	ECHO 8 from brain and lung, Coxsackie B ₉ from feces of patient. Sera obtained from parents did not neutralize isolates from patient.
6. M.T.	10	W	M	Sore throat for 3-4 days pta. Vomiting one day pta. Became delirious and semi-comatose shortly pta. On admission did not respond to oral communication, withdrawn and rotated head upon physical stimulation. Pupils dilated but responded to light. TM's injected and pharynx inflamed. Patient rapidly became decerebrate. He died 13 hours after admission.	WBC - 8,100 - 76% segs CSF - 4 WBCs, Sugar-92 mgm% Protein-70 mgm% Spinal fluid culture-negative	Evidences of infection were lacking in the heart, lungs, adrenals, meninges, anterior horns, and spinal cord, etc. The only morphologic diagnosis from this necropsy was acute splenitis.	Heart Kidney Liver Brain	Coxsackie B ₉ isolated from brain.
7. H.T.	12mo.	W	M	Six days pta rhinorrhea was noted. Two days pta coughing and coryza. One day pta mild respiratory distress. This was more severe on admission. Immediate transferral to referral hospital was made. Here he appeared moribund, moderate cyanosis and marked respiratory depression were noted. Temperature 103°. Reflexes were hyperactive. Shortly after admission, respirations ceased.	WBC - 9,900 with 80% segs CSF - normal Blood, tracheal and stool cultures were negative, both for bacteria and viruses.	No autopsy.	See laboratory findings.	
8. J.S.	6	W	F	Low-grade fever for three days pta. Then onset of tender muscles in the thighs was noted. Child returned home, however, was alert and playful and went to bed that evening. Her only complaint at bedtime was a headache. Father awakened by a noise during the night. Child had stopped breathing and entire body was cold. Moribund on arrival at hospital. Blood pressure maintained for short time with Solucortef drip but she expired shortly.	WBC - 8,000 with a normal differential	Cerebral edema, bilateral atelectasis with rather marked pulmonary edema, and intra-alveolar hemorrhage. A moderate laryngitis was noted. Rest of autopsy negative.	None	

NO.	AGE	RACE	SEX	CLINICAL SIGNS AND SYMPTOMS	LABORATORY FINDINGS	POST-MORTEM FINDINGS	VIROLOGY FINDINGS		
							Specimens Available	Results	
9.	S.B.	9Mo.	W	M	Rhinorrhea and slight cough 3 days pta - vomiting and low-grade fever. Morning after admission patient could not be aroused. Seemed unresponsive, was transferred to a University hospital. Tonic-clonic movements noted on arrival. Also breathing rapidly, marked chest retraction, palpable liver, hyperactive reflexes. Bright light was avoided. Left pupil larger than right, but both reacted to light. Began having generalized tonic-clonic convulsions on second hospital day and became more rigid, pupils dilated. On 4th day he became apneic and his pulse slowed. He expired on the 5th day.	WBC - 23,000 - 40 polys, 15 stabs and the rest small lymphs CSF - 140 mm. pressure, no white cells BUN-25mg%; Na-141; K-6; Cl-107; CO ₂ -8 meq/L Throat culture - negative Urine culture - negative EEG - abnormal - consisting of a focalization involving the left parietal area as well as what appeared to be a mesencephalic movement.	Acute encephalopathy with anoxic neuronal degeneration, acute bronchitis with minimal broncho-pneumonia, acute pancreatitis with fat necrosis.	Blood, throat swabs	Negative
10.	J.E.	6	W	F	Slight fever, nausea, vomiting 24 hours duration pta. One week prior to this had had a sore throat treated with antibiotics. Began vomiting one day pta and continued despite anti-emetics. Shortly after admission began convulsing. Respirations began to be labored and suddenly ceased. Emergency tracheotomy and respiratory apparatus maintained patient until her death 18 hours after admission.	WBC - 14,900 with 63% segs CSF - 1 WBC, Sugar-89mg%; Cl-132meq/L Protein-44mg% BUN-17.9; CO ₂ -10meq/L Tracheal aspirant cultured negative. Blood culture - negative Spinal fluid culture - negative	Marked brain swelling, no evidence of inflammatory change, moderate fatty hepatomegaly, basilar pulmonary hyperemia and edema, renal cortical pallor.	None	
11.	D.L.C.	6	W	F	Child had a "cold" for week pta - treated with aspirin. Onset of fever one day pta with some quivering, shaking and anorexia. On day of admission she complained of pain in her chest, cough and didn't seem to recognize her mother. She later vomited. On admission she was semi-comatose but could be roused by painful stimuli. Neurologic exam and optic fundi were negative. Temperature 101.6°. Morning after admission, generalized convulsion with twitching of the arms and legs was noted. Respirations became very labored. Tonic-clonic movements began and continued until her death five hours later.	WBC - 26,400 segs 65% CSF - 58 white cells per cubic mm with 88% polys and 11% lymphocytes noted on spinal tap; Protein - 61 CO ₂ -15; Cl-106; K-51; Na-132meq/L Spinal fluid blood culture - no growth	Largely limited to the brain. Lymphocytic and hemorrhagic infiltration of the perivascular (Virchow spaces). Pathologists impression: influenza encephalitis.	None	
12.	T.T.	1	W	M	Coughing and fever intermittently for one week pta. Anorexia for the day pta. Listlessness and twitching 4 hours pta. Admission temperature 101°. Responded only to painful stimuli, weakness of left arm and leg were evident--head was turned to right. Neck flaccid, lungs filled with moist rales in the lower lobes, hyper-reflexia was noted. Twitching and semi-comatose state continued intermittently for next 4 days. On fifth day, left hemiplegia noted, on sixth day inability to swallow and tonic-clonic movements continued. On ninth day heart sounds irregular, respiratory depression noted and child died shortly thereafter.	WBC - 22,300 with 89% segs CSF - cells 0, Protein-19mg% Sugar-80mg%; Pressure - 280 mm/ 340 mm on separate occasions	Autopsy not granted.		

NO.	AGE	RACE	SEX	CLINICAL SIGNS AND SYMPTOMS	LABORATORY FINDINGS	POST-MORTEM FINDINGS	VIROLOGY FINDS		
							Specimens Available	Results	
18.	G.D.	B	W	F	Patient a juvenile diabetic, normally under good control. One day pta started vomiting. Vomited all day, retaining nothing. She was talkative and hyper-irritable at admission. Diabetic acidosis was treated. Approximately 8 hours after admission, although acidosis seemed to be under good control, respirations became very slow and she suddenly stopped breathing. Fever to 103. She was maintained on a Bennett respirator for 4 days, along with supportive therapy. She died shortly thereafter, however.	Blood sugar - 500mg% on admission, 300mg% 6 hours after adm. "electrolytes normal" on second day of hospitalization CSF - within normal limits	Brain extremely soft and edematous, swelling throughout. Focal hemorrhages were noted within the pons and mid-brain. No purulent meningitis or abscess was present. Mild bronchitis and peri-bronchitis was noted. Otherwise, autopsy findings not remarkable.	Brain Spinal fluid Lung Liver Stool Spleen Throat washings and stool specimens obtained from the pt's siblings.	ECHO 8 obtained from the pt's feces and lung. Also obtained from throat washings of mother, 1 sibling and from stools of two other siblings.
19.	D.P.	18Mo.	W	F	Child found in crib with open bottle of APCs. Three and one-half hours later was noted to be lethargic and unstable on her feet. Had had a mild upper respiratory infection and low-grade fever for two days previous to this incident. Admitted to the hospital where exam revealed lethargic infant with respiratory rate of 40 per minute. CO ₂ was 18.4 meq and salicylate level reported as normal. Child appeared much improved after fluid treatment. Also, electrolytes returned to normal after fluid treatment. However, 36 hours after admission a generalized convulsion occurred. Reflexes hyperactive, then disappeared. The patient expired 42 hours after admission after progressive respiratory depression.	WBC - 25,800 with 68% neutrophils CSF - no significant abnormality CO ₂ - 14.4; Na-134; Cl-110meq/L Blood salicylate level - within normal limits on two occasions	Marked cerebral edema. Liver was deep yellow in color although histologic study failed to reveal anything remarkable. Terminal edema and vascular congestion noted.	None	
18.	B.M.	4Mo.	C	F	7-10 days pta - upper respiratory infection. 3 days pta - drainage from ear. 1 day pta irritable, vomiting. On adm. temperature 99, comatose and unresponsive to pain. Respirations rapid, pupils reacted sluggishly. Hyperactive deep tendon reflexes. Cheyne Stokes respiration began. Hypertonic dehydration was corrected. Generalized convulsions began. Died 3 days after admission.	WBC - 10,800, 51% PMN's CSF - No WBC's, Sugar - 65mg% BUN-26mg%, CO ₂ -16.3 meq/L, Na-155, K-8.7	Necrosis, early, neurons of hippocampus, acute otitis media, interstitial pneumonia, acute bronchitis.	From patient No results to date. Stool specs. from 5 sibs and parents.	ECHO 8 isolated from stools of 2 sibs and 1 parent. Polio 1 isolated from stool of one sib.
18.	D.W.	13	W	M	One day pta - nauseated with headaches, generalized aches and pains. Somewhat confused the following morning. On admission, talkative, throat was red, stiff neck with sluggish reflexes. Became incoherent. Next day emesis of coffee ground material. Temp. 103. Reacted only to pin-prick. Pupils dilated but reacted sluggishly. Despite tracheostomy, became cyanotic. 3 days after admission seizures occurred. Remained semi-comatose until expired 10 days after admission.	WBC - 25,750, 65% segs CSF - 91 cells (lymphs 58%, monos 20%) protein - 52 mgm.% Na-147 meq/L, K-3.7, CO ₂ -25 Blood culture and CSF culture - neg.	Findings largely localized to the brain. Brain of marked red-purple color. Almost jelly-like in consistency. Hemorrhages (petechial to few mm. in size) throughout. Marked perivascular hemorrhages throughout. Little, if any, evidence of meningeal reaction.	Urine, throat washings, nasal swab	ECHO 8 isolated from urine.

had three features commonly associated with infectious encephalitis.

There were no remarkable bacteriologic findings. In 8 of the 16 cases materials for virologic study were available from the patients, and in 1 case specimens from the family were available. In three cases ECHO 8 was isolated from the brain, lung, feces, and urine respectively. In one case Cocksackie B₄ was isolated from the brain. In one case ECHO 8 was demonstrated in the brain and the lung, and Cocksackie B₄ in the feces. In the case in which ECHO 8 was isolated from the lung and feces it was also isolated from the throat washing of the mother and one sibling, and from the stools of two other siblings. In one case in which no virologic specimens from the patient were available, ECHO 8 was isolated from the stools of two siblings and one parent, and polio 1 was isolated from the stools of another sibling. In the 2 cases in which sera from either the patient or the family were available, we could not demonstrate neutralization of the virus isolated.

Virology Technique

Specimens collected for virologic studies included stools, swabs, blood, urine, cerebrospinal fluid, and tissues taken at autopsy.

Processing of specimens: Stool and tissue specimens were extracted to make a 20 per cent suspension in neutral Hank's solution. The suspensions were centrifuged and antibiotics were added to the final supernatants. Swabs were shaken vigorously in 2 ml. of the same diluent. Fluid specimens were processed by addition of antibiotics. All specimens, extractions, and cultures were stored at 20°C.

Isolation and identification: Each specimen (0.2 ml.) was inoculated into three tubes each of Rhesus kidney (MK) and Hela Gey (H) tissue culture. A growth of media used for MK cell culture consisted of 5 per cent calf serum, 0.5 per cent lacto albumin hydrolysate in Hank's base, and for Hela, 10 per cent calf serum in Media 199, both with antibiotics. The cell cultures were inoculated as soon as a monolayer was established and the growth media exchanged for

maintenance media (same as growth except for MK 2 per cent calf serum and for H 4 per cent calf serum was used).

These cultures were incubated at 36° C and examined each day for an eight-day period. Tubes showing cytopathic effect were harvested when 75-100 per cent of the tissue was disrupted. Tubes of culture were frozen overnight, thawed, cooled, and stored. Each of the specimens was inoculated into 24 hour old mice. Each specimen that was suitable was inoculated into egg embryo by the amniotic route. All viral isolations were identified by neutralization tests in monkey kidney.

Six to nine months later, tests of identification of these viral isolates were repeated. At the same time re-isolation was attempted using the original extractions of the specimens included in the study. The isolates obtained from this re-isolation procedure were also identified by neutralization tests in monkey kidney.

Discussion

It is well recognized that a serious perplexing clinical problem is posed by the infant or child who becomes acutely ill with fever, stupor, or coma and convulsions. Recently, Lyon, Dodge, and Adams⁸ have recognized the difficulty in terminology and differential diagnosis in children with this sort of nervous syndrome. They identify these clinical states as being distinguished by generally negative laboratory results and refer to them as acute encephalopathies of obscure origin. These are the syndromes which are associated with febrile illness, generalized convulsions, and neurologic disorders which occur without evidence of spinal fluid changes and are associated with swelling of the brain as a prominent finding.

Flewett and Hoult⁴ divided into four groups a collection of cases in which influenza virus has been associated with neurologic disturbances. The most dramatic group initially presented confusion and flaccidity, and showed little response to stimuli three days to two weeks following influenza. Their diagnosis was based on commonly accepted laboratory data.

Eli Gold and others⁵ recently studied the

entire problem of sudden deaths in infants. Among other things, they investigated a group of deaths which were associated with the isolation of Coxsackie virus, Group A₄. Even though histologic confirmation was lacking in these cases, the authors felt that their studies raised important questions concerning the pathogenesis of enterovirus infections and pointed out the need for further study.

It has been interesting to see the continuing evidence of a wide variety of clinical manifestations of Coxsackie and EMCO virus disease in infants and children. The serious nature of these illnesses is becoming more and more apparent. Current⁶ and McAllister⁷ have classified Coxsackie and ECHO virus by their association with symptomatic disease. Coxsackie B₄ has been associated with pleurodynia, aseptic meningitis, paralysis, myocarditis, and meningoencephalitis of the newborn, pericarditis, and undifferentiated febrile disease. ECHO 8 has been associated with diarrheal and respiratory disease. To our knowledge, there have been no series associating fatal encephalitic disease with Coxsackie and ECHO virus other than in infants. Recently, Walker and Togo⁸ reported a case of acute diffuse encephalitis associated with isolation of Coxsackie B₂ virus in a 9 year old child. They cite cases of encephalitis in a 16 year old boy, a 22 year old woman, and a 54 year old woman who demonstrated encephalitis-like disease due to Coxsackie B₄ and B₂. They also make reference to a case of fatal meningoencephalitis which was considered to be due to Coxsackie, Group A₄. Sabin⁹ has attributed encephalitis in older people to ECHO type 9.

Considering the review of literature, what then is the true relationship of the viruses isolated in 5 of our 16 cases with the true cause of disease in each case? If there is an association, it might indicate that in these 5 cases the virus was the etiologic agent. If this is true, then might it not also be true that the other 11 cases represent a similar type of disease in which the viruses were not isolated? The obvious alternative is that the virus isolations are coincidental and possibly unrelated to the true cause of dis-

ease. If enteroviruses do play a role in the etiology of fatal encephalitic disease, we feel that this series brings the question into sharp focus.

What then should be done to clarify this situation? We believe that it is imperative that every physician who faces such a problem should make every effort to identify the possible etiology. This effort should include intensive study of each case, allied with all available laboratory techniques, so that a collective study and analysis of such cases may reveal the true cause of this type of disease. This, of course, would mean that we cannot limit our investigation to the child, but must extend it to the family as well. Surely as we progress we must utilize methods of communication so that episodes such as we have experienced can be properly correlated with the experience of other investigators throughout the country.

Summary

We have presented the information which accumulated during the study of 16 deaths due to encephalitis-like disease in North Carolina. It is our hope that others will share our experience, that this presentation will add to the literature, and that it may contribute to the eventual clarification of the etiology of such cases.

Acknowledgement

The authors wish to thank the many people who volunteered their cooperation and supplied the invaluable information presented in this study. They are especially grateful to the physicians who allowed them to use their materials and cases.

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Hernia Adiposa — A Cause of Low Back Pain

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Herniation of subfascial fat has been described as a clinical entity representing displacement of fat from a deep stratum to a more superficial one. This condition was described by Herz¹ 17 years ago. In 1944, however, Copeman and Ackerman² first showed that painful nodules in the lumbosacral regions were actually herniations of fat through the superficial fascia. The chief characteristic of the nodules is a single or multiple mass which the patient is frequently able to localize. The associated pain is usually severe, and the patient is unable to move when first seized by it. The term "fibrositis" is often applied to the condition, since the nodules were originally thought to be caused by excessive fibrous tissue.

In 1955 Herz³ had relieved the symptoms of subfascial fat hernia by operation. Their patients were selected from more than 300 cases of previously undiagnosed low back pain. As a result of his work, we undertook the present study of 20 patients who were referred to the Wendell-Zebulon Hospital Department of Surgery because of low back pain of undetermined cause. All these patients were carefully examined to rule out herniation of nucleus pulposus and other conditions which might have caused their pain.

Surgery was resorted to in those cases where the relief obtained from the injection of the nodules with anesthetic agents and methyl prednisolone acetate (Depo-Medrol, Upjohn) was short-lasting, or when repeated injections were impractical. Many of these patients had been treated for years with various types of support, steroids for arthritic changes of the vertebrae, and so

forth. One of the patients had been operated on for herniation of nucleus pulposus without benefit.

Physical examination included thorough palpation of the lower part of the back to elicit trigger points of pain and painful nodules. In many patients a non-tender nodule in the lumbosacral region was demonstrated. It is felt that this type of nodule could not be responsible for the pain associated with hernia adiposa. Copeman and Ackerman found from an anatomic study of 14 cadavers that the distribution of fat in the lumbosacral region coincided almost exactly with the trigger points of pain in 50 patients.

Operative Technique

After careful attempt to rule out the multiple causes of low back pain, operation was performed on 20 of our patients selected on the basis of either temporary or prolonged relief with infiltration of the above-mentioned agents. The area of herniated fat in the lower part of the back was marked with a dye preoperatively. A skin wheal at this site was produced, by injecting a 10 per cent solution of Zylocaine.

Incision through the skin and superficial fat is followed by evidence of herniation of the underlying fat up to the level of the skin (fig. 1). This displaced fat is quite well defined. Further infiltration with local anesthetic is necessary to dissect it from the underlying fascia. While the dissection is being carried out, the area in the fascia through which course the sensory nerves and blood vessels supplying this fat is usually visualized (fig. 2). Small hernias tend to

Special Articles

Reye's Syndrome: Current Concepts

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In 1963, Reye, Morgan and Baral (1) described the clinico-pathologic features of the disease that is now broadly recognized as Reye's syndrome (encephalopathy and fatty degeneration of the viscera). Over the ensuing years, widespread recognition of the disease and its clinical manifestations has occurred. Characteristically, a prodromal illness, most commonly influenza or varicella, is followed in 3 to 5 days by the onset of pernicious emesis. Initially, patients are well-oriented but irritable and lethargic. Some patients have no change in consciousness and remain only lethargic to variable degrees [Grade I and II encephalopathy, Cincinnati Coma Grade (2)] with no progression to unconsciousness. The AST and ALT levels are three to 30 times normal. Serum bilirubin rarely exceeds 1 mg per dl. Serum ammonia concentrations are variable at presentation. Noncomatose patients may have mildly to moderately elevated (2 to 5 times normal) or normal ammonia concentrations. With worsening encephalopathy to a hyperexcitable state (agitated delirium), the patients are intermittently out of contact with their environment (Grade III). Further progression to deeper comatose states characterized by decerebrate and decorticate posturing, hyperventilation and hyperpyrexia (Grade IV) and finally flaccid paralysis with loss of involuntary ventilatory control (Grade V) may be variable and span only a few hours to a few days. Comatose patients uniformly have elevated venous ammonia concentrations ranging from 3 to 20 times normal. The encephalopathy typically persists for 24 to 96 hr, with gradual improvement in survivors. Recovery of consciousness in patients with permanent neurologic impairment may require weeks.

Voluntary reporting of cases to the Centers for Disease Control (CDC) requires fulfillment of the criteria for the case definition of Reye's syndrome: (i) acute noninflammatory encephalopathy documented clinically by an alteration in consciousness, and, if available, cerebrospinal fluid containing less than 8 leukocytes per mm³; (ii) hepatopathy documented by liver biopsy or autopsy or a 3-fold or greater rise of AST, ALT or serum ammonia, and (iii) no more reasonable explanation for the cerebral or hepatic abnormalities (3).

Percutaneous liver biopsy will confirm the diagnosis of Reye's syndrome; however, the hepatic lesion is eva-

nescent so that biopsy must be performed during the acute phase (the first 3 to 4 days) to find the characteristic histologic, histochemical and ultrastructural alterations. Hepatocytes are usually swollen, an inflammatory infiltrate is absent, abundant panlobular small droplet fat is identified and typical mitochondrial abnormalities are found (see below).

During the last 22 years, over 3,000 cases have been reported to the CDC with a case fatality rate varying from 26 to 42% (4). Therapy for the disease is largely empiric, since its specific pathophysiology remains incompletely defined and its cause remains unknown. The purpose of this review is to acquaint the reader with newer findings regarding the epidemiology, pathogenesis, etiology and animal models of Reye's syndrome. The reader is referred to other reviews for more comprehensive discussions of the clinical, biochemical, histopathologic and therapeutic aspects of the disease (5-8).

EPIDEMIOLOGY

In 1968, the CDC established a voluntary surveillance system for Reye's syndrome. From 1967 to 1973, between 11 and 83 cases were reported annually. Between 1974 and 1983, an increase in reporting frequency was observed with 236 cases reported in 1978-1979 to a peak of 555 in 1979-1980. Thereafter, there has been a steady decline in cases through 1985 when 91 cases were reported. Over recent years, there has been a trend toward diagnosis in earlier coma stages (9). Nationwide, the incidence of Reye's syndrome has ranged from 0.15 to 0.88 cases per 100,000 children under age 18 years. Regional differences in disease incidence are recognized. Pacific states, Nevada, most South Atlantic and East South Central states have consistently low rates while several Mountain, West North Central and East North Central states have consistently high rates (3). During a 5-year retrospective study in Ohio from 1973-1977, the incidence rate was 2.8 to 4.7 cases per year per 100,000 population less than age 18 years in the absence and presence of influenza B epidemics, respectively (10). During prospective surveillance in Michigan during 1973-1974, an incidence of 2.4 cases per year per 100,000 population was observed during a 7-month period which included an influenza B epidemic (11). A recent prospective study in Cincinnati yielded similar results. In 1980-1981, when influenza A was prevalent, an incidence of 3.5 liver biopsy-proven cases of Reye's syndrome per 100,000 population less than age 17 years was found. If CDC criteria had been utilized, a higher frequency of 5.6 cases per 100,000 population under age 17 years would

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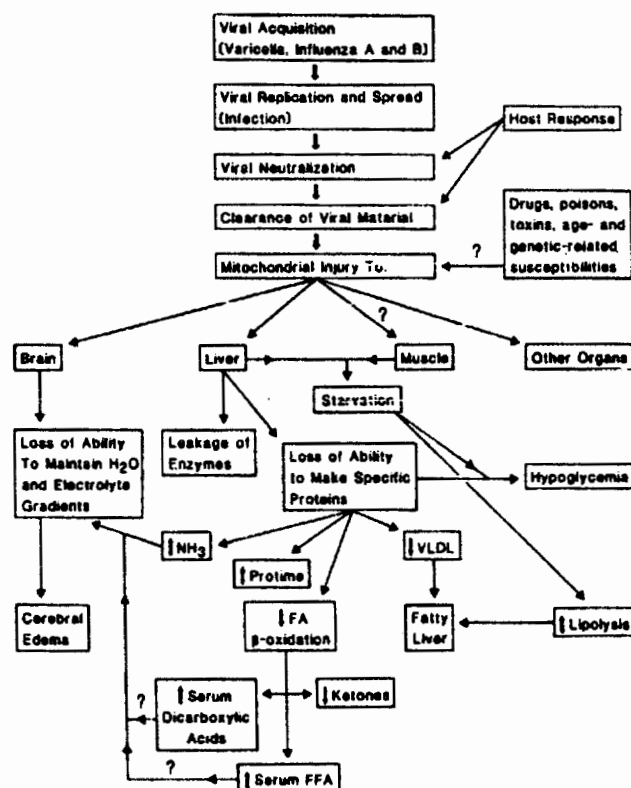
have been observed. Noncomatose cases accounted for greater than three-fourths of all cases, suggesting that the prevalence of noncomatose cases is likely to be much higher than previously recognized (12). Despite high awareness and aggressive surveillance efforts since 1980–1981, the total number of cases have continued to decline in Cincinnati (13). In 1985–1986, only one comatose and one noncomatose case were identified. This parallels the striking decline in cases noted nationwide. This observed decline is quite reminiscent of the experience in New Zealand. After an epidemic of Reye's syndrome in the late 1950's and early 1960's, the disease has virtually disappeared. The reason for this decline in incidence in New Zealand remains unresolved.

PATHOGENESIS

Despite intensive study, the pathogenesis of Reye's syndrome remains incompletely defined. After an initial viral prodromal illness, pernicious vomiting develops after a latent period of 3 to 5 days. The onset of emesis is generally considered the first sign of encephalopathy in Reye's syndrome. Early in the course, sensorial changes may be limited to lethargy (Grade I, Cincinnati Coma Grade) despite protracted emesis, marked elevation in hepatic enzymes (AST, ALT), prolonged prothrombin time, increased free fatty acid concentrations and normal or modestly increased serum ammonia concentrations. Intracranial pressure, as assessed by lumbar puncture, is generally normal in coma Grades I and II. With increasing neurologic alterations including deeper lethargy (Grade II) and agitated delirium (Grade III), increasing metabolic derangements are commonly noted. Serum free fatty acid concentrations rise and dicarboxylic acids appear in urine and serum, and the serum ammonia is markedly increased. Co-incident with these changes, the intracranial pressure may become elevated. With deepening encephalopathy, increase in intracranial pressure commonly precludes effective cerebral perfusion, leading to brain damage or death.

A series of cascading metabolic events occurs during the course of Reye's syndrome. It remains unclear whether the pathogenesis can be explained by a primary injury to the mitochondria of multiple organs including the brain, liver and muscle with its metabolic consequences or whether a primary hepatic injury leads to metabolic consequences which produce the biochemical abnormalities and encephalopathy. Based upon experience with noncomatose cases, it appears likely that the encephalopathy (vomiting and lethargy) is present in most cases prior to the appearance of significant hyperammonemia and markedly increased serum-free fatty acids. However, hyperammonemia, acid-based disturbances, hypoglycemia, free fatty acidemia, dicarboxylic acidemia and salicylates may have some synergistic effects with the primary mitochondrial injury and lead to the development of life-threatening cerebral edema (Figure 1).

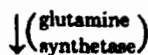
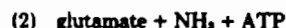
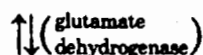
Morphologic and biochemical studies have confirmed the presence of a characteristic mitochondrial injury. Pleomorphic, enlarged mitochondria with disrupted cristae, electron lucent matrices and reduced dense bodies are characteristic of the hepatic pathology of Reye's



lopathy of Reye's syndrome. However, since neurons comprise a small proportion of all brain cells, failure to demonstrate reduced mitochondrial enzyme activity is not totally unexpected (20; Partin, J. S., *Pediatr. Res.* 1979; 13:868, Correspondence). Two concurrent mechanisms of cerebral injury may be present. A primary injury is suggested by the mitochondrial lesion while co-existent astrocyte swelling may suggest injury secondary to hyperammonemia.

The biochemical derangements present in Reye's syndrome, alone or in combination, may be responsible for the pathogenesis of the disease. Hyperammonemia results from excess nitrogen load secondary to protein catabolism and reductions in activity of intramitochondrial enzymes of the urea cycle, ornithine transcarbamylase and carbamyl phosphate synthetase. Hyperammonemia correlates with severity of coma grade of Reye's syndrome and likelihood to progress from noncomatose to comatose states (21-24). The symptom complex observed in children with congenital ornithine transcarbamylase deficiency and hepatic encephalopathy is similar to Reye's syndrome. Vomiting, coma, hyperventilation, astrocyte swelling and increased intracranial pressure are common to all conditions. Studies in young primates have demonstrated that graded infusions of ammonium acetate lead to progressive alteration in consciousness and increased intracranial pressure associated with astrocyte swelling but no neuronal pathology (25). Infants dying in hyperammonemic coma have cerebral edema associated with astrocyte swelling while patients dying after recurrent hyperammonemic episodes have cerebral atrophy and the appearance of Alzheimer Type II cells (26). In patients dying with liver failure and portosystemic encephalopathy, gross cerebral edema and swollen astrocytes are commonly encountered (27).

The pathogenesis of ammonia and portosystemic encephalopathy remain incompletely understood. The brain detoxifies ammonia via glutamine formation:



This process deprives the electron transport chain of NADH, leads to reduced oxidative metabolism and reduced stores of high energy phosphates (28). Ammonia increases glycolysis and leads to increased pyruvate and lactate concentrations, reduced glutamate concentrations and increased glutamine synthesis (29, 30). The generated glutamine may accumulate in astrocytes and thereby lead to an osmotic shift in water resulting in cerebral edema (31; Brusilow, S. W. et al., *N. Engl. J. Med.* 1986; 314:786-787, Correspondence). Glutamine concentration in the cerebrospinal fluid has been proposed to be a sensitive measure of hepatic encephalopa-

thy (32). However, serum and cerebrospinal fluid glutamine concentrations are generally normal in Grade I Reye's syndrome when serum NH_3 is normal or mildly increased (personal observation). Hyperammonemia or associated disturbances do not appear to initiate the encephalopathy of Reye's syndrome but may be important confounding factors after hepatic mitochondrial dysfunction leads to hyperammonemia.

Excessive lipolysis from adipocytes with impaired hepatic metabolism of the liberated fatty acids and the panlobular microvesicular fatty change is characteristic of all coma grades of Reye's syndrome. Elevated serum-free fatty acids are present even in mild cases, but concentrations are not directly proportional to severity of coma grade (33-36). Coincident with the increased free fatty acid concentrations, patients tend to be ketopenic, and dicarboxylic acids appear in urine and serum. These findings suggest that mitochondrial β -oxidation of fatty acids is compromised, and alternative extramitochondrial routes of oxidation are utilized. The serum pattern and the urinary excretion of dicarboxylic acids suggest that medium chain acyl CoA dehydrogenase enzyme activity may be reduced. Additional studies have suggested that a "relative carnitine deficiency" is present which precludes efficient shuttling of fatty acids across the mitochondrial membrane prior to oxidation. Theoretically, carnitine deficiency would lead to the accumulation of toxic acyl CoA derivatives (37, 38). Concentrations of hepatic short-chain CoA esters are increased while long-chain CoA esters were comparable to controls (39). These results suggest that the short- and medium-chain acyl CoA dehydrogenase enzymes may be reduced, but long-chain acyl CoA dehydrogenase activity is intact and the carnitine "shuttling" system is intact. In addition, no studies have demonstrated that serum total or free carnitine concentrations are reduced despite apparent loss of large quantities of free and acylcarnitines in urine and low hepatic concentrations in children with Reye's syndrome (40-43; Hinshaw, W. B. J. et al., *N. Engl. J. Med.* 1980; 302:1423, Correspondence; Roe, C. R. et al., *J. Natl. Reye's Syndrome Found.* 1983; 4:58, Abstract; Lansky, L. L. et al., *Pediatr. Res.* 1985; 20:391, Abstract). The role of fatty acids in the pathogenesis of Reye's syndrome has been the subject of debate for over 10 years. Concentrations of serum-free fatty acids increase during starvation with no sensorial change; levels attained may exceed those found in Reye's syndrome (44). Infusions of short-chain fatty acids (octanoate) into animals may lead to encephalopathy and mild elevations of ammonia and lactate and increased intracranial pressure (45, 46). Fatty acids of varying chain lengths are capable of uncoupling oxidative phosphorylation and inhibiting β -oxidation in isolated liver mitochondria (47, 48). Furthermore, addition of albumin to the incubation medium may lead to ablation of the effect on isolated mitochondria (49). Nonesterified fatty acids can induce mitochondrial swelling *in vitro* and reduce mitochondrial enzyme activities, including carbamyl phosphate synthetase (50). Anaevin has suggested that short- and medium-chain fatty acids may alter brain mitochondrial function. When rat brain mitochondria were incubated with serum from patients with Reye's syndrome, State 4 respiratory

rates were increased and the respiratory control ratio was decreased. The observed alterations were dose-dependent and blocked by preincubation of serum with fatty acid-free albumin (51). Inhibition of fatty acid oxidation in brain capillaries by agents like pantoic acid may alter active ion transport and lead to development of cerebral edema (Goldstein, G. W., *N. Engl. J. Med.* 1977; 296:632-633, Correspondence). Fatty acids may inhibit brain $\text{Na}^+\text{K}^+\text{-ATPase}$ with inhibitory capacity directly proportional to fatty acid chain length and concentration (52). This may interfere with ion pumping and lead to alterations in sensorium.

Both fatty acids and dicarboxylic acids may have profound effects on mitochondrial morphology and function (Picardo, M., *J. Invest. Dermatol.* 1982; 80:350, Abstract). Serum from patients with Reye's syndrome increases oxygen consumption, uncouples oxidative phosphorylation, impairs ATP formation and causes swelling of chinchilla mitochondria (53). Dicarboxylic acids of $\text{C}_6\text{-C}_{10}$ chain length have been identified in urine, and increased concentrations of other short-chain dicarboxylic acids have been found in serum of patients with Reye's syndrome. Recent studies have shown that as much as 54% of total serum-free fatty acids are present as dicarboxylic acids with 90% being $\text{C}_{16}\text{-C}_{18}$ chain length (54, 55). Dicarboxylic acids are commonly encountered in urine from patients with inborn errors of metabolism involving the β -oxidative pathway (56-58). In addition, small quantities may be encountered in children with diabetes mellitus and protein calorie malnutrition (59). Dicarboxylic acids, including sebacic, suberic and adipic acid in quantities similar to those found in Reye's syndrome, may be present in the urine of infants treated with medium-chain triglyceride formula without apparent ill effect (60). However, in no other condition have long-chain dicarboxylic acids been found in concentrations comparable to those found in Reye's syndrome (61; Tonsgard, J. H., *Pediatr. Res.* 1986; 20:468, Abstract). Whether the appearance of dicarboxylic acids in the serum are secondary to mitochondrial dysfunction or are intimately involved in the pathogenesis of Reye's syndrome remains undefined at the present time. Additional studies will be needed to better define their role.

The role of salicylates in the pathogenesis still remains unclear. Salicylate usage commonly precedes the onset of Reye's syndrome. Serum salicylate concentrations are increased in patients with Reye's syndrome compared to community-based controls; however, no correlation has been found between coma grade and serum concentration (62). Alterations in salicylate metabolism may accompany Reye's syndrome. Aspirin esterase activity may be reduced and salicylate turnover rates prolonged in patients with Reye's syndrome (63; Tomasova, H., *Lancet* 1984; 1:43, Correspondence). Salicylate intoxication produces severe acid-base disturbances, coagulopathy, hepatic dysfunction and encephalopathy. Children who died after salicylate intoxication have been found to have cerebral edema and intrahepatocyte microvesicular change without necrosis or inflammation (64). These findings were felt to indicate that the histologic findings in Reye's syndrome and salicylate intoxication were similar. Recently, microvesicular fatty change has been ob-

served by Bunnell and Beckwith (65) in children who died after cranio-cerebral trauma, suggesting that the microvesicular fatty change observed at autopsy may be somewhat nonspecific. Review of the autopsy series at Children's Hospital in Cincinnati revealed that 5 of 6 patients dying of single dose salicylate intoxication had vacuolization of hepatocytes without cell necrosis or inflammation (Daugherty, C. C. et al., *Lancet* 1983; 2:104, Correspondence). Careful light microscopic and ultrastructural pathology studies have demonstrated minimal lipid accumulation during salicylate toxicity with normal mitochondrial structure. In contrast, liver pathology findings from patients with Reye's syndrome include prominent microvesicular fat and enlarged pleomorphic mitochondria with expanded matrices and no mitochondrial dense bodies (66). Salicylates uncouple mitochondrial respiration, impair ATP formation and induce mitochondrial swelling (67-69). In isolated rat mitochondria, salicylates and salicyl compounds can potentiate Ca^{2+} -induced damage to the inner mitochondrial membrane (69). Salicylates may: (i) further compromise mitochondrial function directly by uncoupling oxidative phosphorylation; (ii) increase the CoA load to the mitochondrion for oxidation, and (iii) displace bound fatty acids and dicarboxylic acids from serum albumin. Salicylates may serve as an additive factor in the pathogenesis of Reye's syndrome (see below); however, none of these potential mechanisms has been tested or proven.

The role of endotoxin, interferon and tumor necrosis factor in the pathogenesis of Reye's syndrome remain poorly defined, and more work is needed to define their importance. Increased concentrations of endotoxin-like material have been found in comatose children with Reye's syndrome. Levels declined with improvement in sensorium (70). Increased serum lactate, free fatty acids and ammonia with hepatic lipid accumulation and mitochondrial alterations have been observed in starved rats injected with sublethal doses of endotoxin (71). Lymphocytes obtained from children with Reye's syndrome produce less interferon in response to New Castle virus compared to controls (72). Lymphocytes from emulsifier-treated rats given encephalomyocarditis virus respond poorly to agents which induce interferon production (see below). Recently, it has been suggested that enhanced released tumor necrosis factor from macrophages previously exposed to salicylates may contribute to the development of Reye's syndrome (73). Interestingly, tumor necrosis factor release is enhanced to a greater extent by nonsteroidal antiinflammatory agents other than aspirin. To date, no cases of Reye's syndrome have been reported in patients (juvenile rheumatoid arthritis) treated chronically with nonsteroidal antiinflammatory agents other than aspirin. The failure to identify such an association suggests the tumor necrosis factor probably plays a minor role in the pathogenesis of Reye's syndrome.

In summary, based upon available evidence, it appears as if a primary mitochondrial injury initiates multiple metabolic disturbances. Hyperammonemia, free fatty acidemia, lactic acidosis and dicarboxylic acidemia result. Synergistically, the metabolic abnormalities and the underlying mitochondrial injury lead to the observed patho-

physiology through, as yet, incompletely understood mechanisms (74). Fatty acids, dicarboxylic acids, salicylates and other factors (some currently undefined) may inhibit mitochondrial ureagenesis and potentiate their individual metabolic effects. Alternatively, they may inhibit ATP synthesis, and hyperammonemia may deplete cellular ATP and lead to profound reductions in high energy phosphate required to catalyze a myriad of enzymatic reactions. However, no evidence had been presented to confirm that hepatic ATP content is reduced in Reye's syndrome (75).

ETIOLOGY

Despite multiple clues derived from clinical observations, the etiology of Reye's syndrome remains obscure (Figure 1). An antecedent viral illness appears to be an essential ingredient in the development of the disease. In most large studies of Reye's syndrome, upper respiratory illnesses are the prodromal illness in 60 to 70% of cases with varicella present in 20 to 30% and diarrheal illnesses in 5 to 15% (9). Of the implicated agents, influenza A and B and varicella have been most thoroughly investigated. During epidemics of influenza A and B in 1973-1974, 1976-1977, 1977-1978, 1979-1980, and 1980-1981, a temporal relationship between reported cases of Reye's syndrome has been clearly demonstrated (3, 76). Similar seasonal variations in the incidence of varicella and in reported cases of Reye's syndrome have been observed. Infants and children are most commonly affected and until recent years, most affected children with a respiratory prodrome were 8 to 11 years old while the mean age with varicella and gastrointestinal prodromes was 6 years (9). Although rare, well-documented cases of Reye's syndrome have been reported in adults (77). Studies evaluating the role of genetic factors have been largely unrewarding. Although cases have been observed in siblings during outbreaks of influenza, no specific HLA linkage has been identified (76).

TOXINS

Since the first descriptions of Reye's syndrome, investigators have been fascinated by the possible association with toxins. Reye et al. (1) suggested that an environmental toxin might be responsible for the development of the disease. Subsequent investigations have been unrewarding. Exposure to a variety of agents including salicylates, valproic acid, the unripe fruit of the Akee tree, margosa oil, chlordane, pyrrolizidine, camphor, methyl bromides and multiple stings by the Oriental hornet may produce clinical manifestations quite similar to Reye's syndrome (3, 78-85). However, none have had sufficiently similar laboratory, histopathologic or ultrastructural pathologic features to make them serious candidates as the cause of Reye's syndrome.

Exposure to aflatoxin, pesticides and their emulsifiers, paint and aspirin all have been reported in patients with Reye's syndrome (3, 78, 86-91). Aflatoxin B₁ was found in blood and urine in early studies from several children with Reye's syndrome; however, additional work failed to demonstrate any significant difference in aflatoxin concentrations in serum and urine from children with

Reye's syndrome compared to controls (86-89). Based upon clustering of cases of Reye's syndrome in an area of pesticide spraying in Canada and studies from Thailand, a causal association has been suggested (90, 91). Linnemann et al. (78) noted a history of contact with pesticides among children with Reye's syndrome. It was hypothesized that pesticide emulsifiers might alter the pathogenesis of viral infections (potentially mediated through alterations in interferon production) and thereby produce Reye's syndrome (see "Animal Models"). No studies, to date, have successfully shown accumulation of either of these materials in tissues or in the serum of affected patients (92).

SALICYLATES

A relationship between Reye's syndrome and prior salicylate exposure has been proposed for many years. The clinical similarities between salicylate intoxication and Reye's syndrome fueled interest in a potential association. A report of cases of salicylate-treated varicella with encephalopathy which antedated the original description of Reye's syndrome may represent the first indication of a potential link between salicylates and Reye's syndrome (93). Linnemann et al. (78) found that 53 of 56 biopsy-proven cases of Reye's syndrome had prior salicylate exposure (78). Three of the children were receiving aspirin for treatment of juvenile rheumatoid arthritis and one for treatment of rheumatic fever. The first of a series of case-control studies evaluating the potential role of salicylates in Reye's syndrome was published by Starko et al. (94). A significantly greater proportion of children with Reye's syndrome were shown to have aspirin exposure compared to controls. Seven patients had Reye's syndrome based on the CDC criteria (2 of 7 had biopsy confirmation). All seven of the reported cases received aspirin during their antecedent illness while only 8 of 16 age- and sex-matched classmate controls had received salicylates. Unfortunately, this study was seriously flawed by delays in interviews of cases and controls, failure of case verification with liver histology and the absence of blinding of interviewers as to whether patients were cases or controls.

Two additional case-control studies investigated the association between salicylate exposure and Reye's syndrome. The results of the first study conducted in Michigan suggested a statistically significant association between aspirin usage and Reye's syndrome. During 1979-1980, 24 of 25 cases of Reye's syndrome (diagnosed using CDC criteria, histology confirmation unspecified) received aspirin compared to 34 of 46 controls matched for age, race, sex, school grade and antecedent illness. During 1980-1981, 12 of 12 cases of Reye's syndrome received aspirin-containing products compared to 13 of 29 controls (95). The second study was conducted in Ohio from December 1978-March 1980. Ninety-seven cases of Reye's syndrome were identified who fulfilled CDC criteria (19% were confirmed with liver histology) and compared to 156 control subjects matched for sex, race, geographic location, time and type of prodromal illness. Aspirin was ingested by a statistically significantly larger proportion of cases (97%, 94 of 97) than of controls (71%,

110 of 156). No relationship was found between dosage and coma grade (96). Subsequent analyses of both studies suggested that significant methodologic flaws seriously compromised the interpretation of the results. These flaws involved: (i) failure of case definition; (ii) selection of controls; (iii) identification of medications administered to cases and controls, and (iv) parental recall bias and interviewer bias (97).

In response to concerns regarding potential flaws in previous epidemiologic studies, the CDC initiated a prospective study designed to correct these potential shortcomings. The design included multiple ($n = 4$) control populations and allowed inclusion of only subjects with Grade II or greater encephalopathy in hopes of reducing errors in diagnosis; however, liver pathology was not required for entry into the study. Instead, the CDC criteria for diagnosis were utilized. Caretakers of cases and controls were to be interviewed promptly after identification and care was taken to ensure proper identification of medications. Between February and May 1984, a pilot study was conducted to examine the methods to be used for the larger "definitive" study. Thirty patients with Reye's syndrome (seven had biopsy or autopsy confirmation) were compared with 145 controls matched for age, sex, race, antecedent illness derived from hospital, emergency room, school and community populations. Significantly more cases (93%) had received salicylates than members of each control group or all groups combined (46%) during matched antecedent illnesses. Correction for severity of prodromal illness did not change the results. The estimated odds ratio of developing Reye's syndrome after salicylate exposure was calculated to be 16.1 (98).

Two additional lines of study have suggested a circumstantial relationship between Reye's syndrome and aspirin exposure. Recent descriptions of cases of Reye's syndrome among patients with connective tissue disease have implied that this population is at increased risk because of chronic salicylate exposure (99, 100). Remington et al. (101) suggested that aspirin-treated children with juvenile rheumatoid arthritis in Michigan were at significantly greater risk for development of Reye's syndrome than the general pediatric population. Second, the reduced aspirin usage in Michigan has been implicated as the reason for the decline in incidence of Reye's syndrome (102). Results of telephone interviews performed in Techumseh Michigan in 1981 and 1983 indicated that fewer parents gave aspirin for colds in 1983 (25%) compared to 1981 (56%). A parallel decline in the incidence of varicella-associated Reye's syndrome occurred during the same time (29.6 vs. 5.4 cases of varicella associated Reye's syndrome per 100,000 cases of varicella in 1981 and 1983, respectively). Nationwide surveillance and additional surveys conducted in Ohio and Houston have suggested a nationwide parallel decline in aspirin usage and Reye's syndrome (4, 102, 103). Additional factors may have changed in Michigan and nationwide over recent years, but none have been examined.

Reye's syndrome is a rare disease that defies careful, controlled studies. Without an animal model that reliably reproduces the human disease, prospective case-control

epidemiologic studies are the only feasible means to investigate the relationship between medication usage or toxin exposure and Reye's syndrome. However, this type of study cannot establish a causal relationship between xenobiotic exposures and the development of Reye's syndrome. Despite serious evidence incriminating aspirin in the etiology of Reye's syndrome, it is mandatory that physicians realize that the absence of aspirin exposure does not exclude the diagnosis of Reye's syndrome. It is clear that, in all case series, a small proportion of cases do not have aspirin exposure during the prodromal illness. At this time, it appears prudent to recommend that children refrain from aspirin usage during periods of influenza activity and in the presence of varicella as recommended by the Surgeon General and the American Academy of Pediatrics. In addition, parents should be advised about the judicious use of medications including antipyretics for the control of symptoms associated with common childhood illnesses.

INBORN ERRORS OF METABOLISM

In recent years, a number of inborn errors of metabolism have been identified in infants and children who were initially believed to have recurrent Reye's syndrome. Inborn errors of ureagenesis including ornithine transcarbamylase (OTC) deficiency and carbamyl phosphate synthetase deficiency may mimic Reye's syndrome. Affected males with OTC deficiency usually present in the newborn period, and Reye's syndrome is not commonly considered. Heterozygote females with OTC deficiency commonly present with recurrent episodes of life-threatening hyperammonemia, elevated urinary orotic acid excretion, characteristic serum amino acid patterns with elevated glutamine and reduced citrulline concentrations (104). Liver histology may reveal microvesicular fatty change but other mitochondrial enzymes (succinic acid dehydrogenase) are preserved, and mitochondrial morphology is normal or distinctly different from Reye's syndrome (26, 105-107, personal observations). Multiple defects of fatty acid metabolism have been recently identified which mimic Reye's syndrome. Systemic carnitine deficiency, carnitine palmitoyl transferase deficiency, medium-chain acyl CoA dehydrogenase and long-chain acyl CoA dehydrogenase deficiencies commonly present with recurrent episodes of lethargy, seizures, coma and hepatomegaly. Biochemical derangements include hypoglycemia, mild elevation in AST and ALT, hyperammonemia, acidosis, reduced serum carnitine, ketopenia and dicarboxylic aciduria (46, 56, 57, 108-110). A clinical history of recurrent episodes and a family history of affected siblings are commonly elicited. Liver histology may reveal microvesicular or macrovesicular fatty change with mitochondrial changes different from Reye's syndrome (111). Additional organic acidemias including β -hydroxy- β -methylglutaric acidemia and isovaleric acidemia are phenotypically similar to Reye's syndrome (112). The recurrent nature of attacks, young age, family history and disease-specific biochemical perturbations generally suggest metabolic diseases rather than Reye's syndrome in these clinical circumstances.

ANIMAL MODELS

Presently, no available animal model reliably reproduces the biochemical and pathologic features of Reye's syndrome in the human. The role of xenobiotic initiating factors including aflatoxin, insecticides, herbicides and surfactants have been extensively explored in a number of animal species. Many of the histopathologic and biochemical features of the disease have been replicated in these models, but none of the proposed environmental toxins has been identified in significant quantities in the biologic material obtained for patients affected with Reye's syndrome (89, 92). Emulsifiers have been examined for their potential role in the development of a Reye's-like disease in animals. Mortality rates of mice treated with encephalomyocarditis virus and emulsifiers were increased compared to controls. Alterations in hepatic ultrastructural morphology including fatty change, minimal alterations in AST and equivocal changes in hepatic OTC activity were observed (113-115). Emulsifier-treated mice infected with influenza B had increased mortality and serum NH_3 compared to influenza B injected or emulsifier-alone treated mice. Mitochondria from animals treated with emulsifier and influenza B were enlarged with increased matrix lucency, altered cristae and absent dense bodies compared to other treatment groups; however, no hepatocyte fatty change was noted (116). None of these models produced sufficiently similar changes to those observed in Reye's syndrome to be seriously considered as an appropriate model for the disease.

The role of the biochemical perturbations observed in Reye's syndrome in its pathogenesis have been explored using a number of models. Ferrets rendered arginine-deficient and infected with intranasal influenza B have been evaluated as a model of Reye's syndrome. Affected animals had mild increases in AST, markedly increased serum ammonia and encephalopathy. Hepatic ultrastructure alterations included mildly pleomorphic mitochondria with equivocal reduction in matrix density and reduced numbers of dense bodies, increased amounts of lipid and no proliferation of the endoplasmic reticulum or increase in numbers of peroxisomes (117, 118). This model appears more a model of extreme hyperammonemia rather than Reye's syndrome. Increasing free fatty acids in the ferret using a synthetic diet increased hepatic lipid but did not alter the serum ammonia (119). Aspirin treatment of influenza B-infected arginine-deficient ferrets increased mortality, serum NH_3 , AST and hepatic lipids while hepatic OTC was reduced. However, similar biochemical alterations also appeared in control animals (120). Rabbits given intracisternal, unsaturated long-chain fatty acids developed rostral-caudal dose-dependent progression of neurologic abnormalities, but the AST only rose modestly after the appearance of neurologic dysfunction. Pretreatment of animals with aspirin reduced the severity of the neurologic changes (121).

Two recently developed models offer promise as useful models of the disease. Davis et al. (122) have successfully infected 3- to 4-week-old Balb/c mice with a large dose of intravenous influenza A/Lee virus. The animals de-

veloped lethargy, coma and seizures. The AST rose markedly while ammonia was only modestly increased. Hepatic microvesicular fatty change without inflammation was present with only occasional mitochondria having matrix expansion while mild cerebral edema without inflammation was found (122, 123). This model closely approximates the human disease but two concerns are raised: (i) the large inoculum of virus required to produce the disease by the intravenous rather than via the respiratory tract, and (ii) the failure to consistently show the mitochondrial changes typical of Reye's syndrome and reduction of succinic acid dehydrogenase activity (a mitochondrial enzyme) to $\leq 50\%$ in only 9 of 13 mice. Additional studies using this model have failed to show increased mortality in aspirin-treated mice despite serum concentrations as high as 68 mg per dl (124). Brownstein et al. (125) have reported the spontaneous appearance of a Reye's syndrome-like illness in Balb/cByJ mice 4 to 33 days after introduction of animals to rooms with indigenous murine viruses. The illness typically lasted 24 hr and was characterized by rapidly deteriorating consciousness and ultimately death. The serum ammonia was markedly elevated ($38 \times$ normal) in affected mice compared to controls. In 3 of 5 outbreaks, involving 66% of animals, an active coronaviral enteritis was present. Pathologic findings were strikingly similar to human Reye's syndrome. In the brain, astrocytes were swollen without inflammation. Liver pathology findings included microvesicular fatty change, presence of pleomorphic, electron lucent mitochondria with reductions in dense bodies and intact cristae, and increased numbers of peroxisomes. Unfortunately, Brownstein et al. have been unable to reproduce this model, which may seriously hamper its future use (personal communication). Although the development of the models of Davis and Brownstein offered promise, serious drawbacks in both compromise their capability to improve our understanding of the etiology and pathogenesis of Reye's syndrome (126).

SUMMARY

Despite greater than 23 years of study, an incomplete understanding of the etiology, epidemiology and pathogenesis of Reye's syndrome persists. Better understanding of the disease has been hampered by the lack of a good animal model on which hypotheses of its pathogenesis could be tested. Human studies indicate that a primary mitochondrial injury may lead to complex metabolic disturbances that produce the observed pathophysiology. Specific directions regarding avenues for future research should pursue two lines: (i) a good animal model still needs to be developed in which the biochemical and morphologic alterations identified in Reye's syndrome are duplicated. This model should include an antecedent viral illness but may not require aspirin exposure as an essential ingredient. With the identification of a satisfactory model, specific questions about the roles of environmental toxins or medications may be answered. (ii) Study of noncomatose cases of Reye's syndrome should continue. The specific emphasis should be to delineate what factors (NH_3 , free fatty acids and

dicarboxylic acids) may be implicated in the pathogenesis of the CNS disease with the hopes of devising strategies for more effective treatment of encephalopathy and its attendant morbidity and mortality.

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Reye Syndrome

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Reye syndrome is an uncommon neurologic disorder with an annual incidence ranging from 0.3 to 6.0 cases per 100,000 children.^{18,24,60} These statistics are influenced by the periodic coincidence of influenzal epidemics. When first described in 1963, it was considered to be a rare and often devastating illness of childhood.^{51,85} There is little doubt that the illness antedated these two reports,^{8,16} but the earlier literature is sketchy and anecdotal. Pathologists overlooked (or dismissed as unrelated) the fatty degeneration of the liver and other viscera, and liver function tests were not readily available for clinical use. Consequently, these earlier cases were classified as acute encephalopathies⁶⁴ or encephalitides.^{1,63}

This clinical entity is now recognized throughout the world.^{20,26,85} It is a major cause of noninfectious neurologic death following a viral illness in the pediatric age group; and despite the fact that it is an uncommon disease, it still represents one of the primary causes of death for young children in the United States. The dramatic nature of the presentation, the possibility for complete recovery, and the relative frequency of the illness have encouraged physicians and scientists to investigate this illness in some detail. This increasing level of awareness and interest has been paralleled by an apparent reduction in the annual morbidity and mortality.

CLINICAL MANIFESTATIONS

Children of all ages may be at risk for developing Reye syndrome. Occasional cases have been described in early infancy⁴⁹ and in adults.^{105,115} There is no sex preference. The epidemic forms of the syndrome usually parallel the annual incidence of influenza B or A; the sporadic cases typically follow varicella. Other viral agents also have been implicated,^{15,26} and occasionally the illness may develop after bacterial pharyngitis.

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There is no clear correlation between the intensity of the antecedent viral illness and the encephalopathy that follows. Often, the child is recovering from the viral illness when pernicious vomiting begins. Occasionally, the antecedent viral illness is appreciated only in retrospect after the encephalopathy has passed. The encephalopathic phase of the illness is heralded by irritability, lethargy, and vomiting; occasionally fever may be present.

Vomiting is the cardinal clinical manifestation introducing the second phase of the illness. The mechanism underlying the vomiting remains unexplained. Campbell has suggested that elevated polyamines, and specifically spermine, may be the mechanism causing the vomiting.¹³ He cites the experiments of Risetti and Mancini who noted pernicious vomiting in human volunteers after the intramuscular administration of spermine.⁸⁶ The child often will recall the early phase of vomiting after recovering from the encephalopathy, suggesting that cerebral function and specifically memory remains preserved at this point in the illness. Some patients experience no demonstrable neurologic disturbance and they are suspected of having Reye syndrome only because of chemical evidence of hepatic dysfunction. Other less fortunate children are plunged into coma after a few hours of pernicious vomiting. The diagnosis may be unclear in selected cases because of the marked variability of the encephalopathic phase. Typically, however, the patient enters a hyperexcitable state (toxic delirium) after the initial period of vomiting. Such a patient is extremely restless and vigorously resists restraints. The hyperexcitable state is associated with sympathetic nervous system overactivity manifested by fever, sweating, tachycardia, pupillary dilation, and tachypnea. Catecholamine (norepinephrine, epinephrine, and dopamine) concentrations may be elevated in the blood and cerebrospinal fluid (CSF), and the degree of elevation may correlate with the patient's stage at the time of admission.³²

The encephalopathy characteristically lasts for 24 to 96 hours, and complete recovery of organ function is expected in patients who survive. The quality of survival is determined by many factors including hypoglycemia, cerebral hypoxia, hyperpyrexia, hyperammonemia, free fatty acidemia, systemic hypotension, and intracranial hypertension. Other devastating complications include gastrointestinal hemorrhage, pancreatitis, focal or generalized convulsions, and intractable brain swelling. The intensity of these complications determines the quality of survival. A full recovery can be anticipated in almost every case, despite severe neurologic dysfunction during the height of the acute encephalopathy.

The stage of the illness upon admission correlates with the likelihood of recovery.^{48,60,94} Several staging criteria have been promulgated over the past decade including the original staging method outlined by Huttenlocher.⁴⁵ We continue to use this four-stage method. Others subscribe to the five-stage criteria outlined by Lovejoy and colleagues⁶² or the five-stage criteria resulting from the National Institutes of Health Consensus Conference.¹⁶ The

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Glasgow coma scale also has been used for the evaluation of level of consciousness in children with Reye syndrome.³⁰ Duncan and colleagues concluded that the Glasgow coma scale provided better earlier indication of progressive central nervous system (CNS) disease than the Lovejoy scale, and therefore may be of greater value to physicians caring for such patients. Whether one chooses the Huttenlocher, the Lovejoy, or the Glasgow coma scale, it appears that the common denominator is a well-defined set of criteria that enables the examining physician to assess the severity of the illness at the time of admission and to reassess the patient's condition serially as treatment is being administered. Any of these scales is satisfactory, particularly if the examining physician is familiar with the criteria.

Children admitted in the milder encephalopathic stages almost always do not progress before making a full recovery. Conversely, patients admitted in a comatose state frequently deteriorate further while in hospital before recovering completely or dying. The patients at greatest risk of dying are those admitted in deep coma with decerebrate posturing. These observations suggest that early diagnosis and treatment of the child with Reye syndrome aborts the progressive aspect and facilitates recovery. This conclusion, however, remains unproven, particularly as an increasing number of children with mild disease are being diagnosed. This has also contributed to the lower morbidity and mortality that has been noted in recent years.

LABORATORY ABNORMALITIES

The most significant abnormalities are elevated serum transaminases, hypoprothrombinemia, and hyperammonemia. Other serum enzyme abnormalities include elevated activities of creatine phosphokinase, lactic dehydrogenase, amylase, lipase, and glutamate dehydrogenase. The elevated glutamate dehydrogenase activity is masked by a dialyzable inhibitor.⁴⁶ This inhibitor may be GTP.⁴⁵ Experiments have shown the glutamate dehydrogenase activity in Reye syndrome sera to be inhibited about 1000-fold more potently by GTP than is the case with normal human enzyme; and that ADP, which normally reverses the GTP inhibition, has no effect on the aberrant enzyme present in the sera of patients with Reye syndrome.⁴⁵

The serum glucose and phosphorus concentrations may be decreased, the uric acid level increased, and the carbon dioxide level decreased. The arterial pH, however, frequently is in the normal range despite the hyperpnea and the organic acidemia.²⁷ These abnormalities are consistent with a mixed acid-base disturbance, each component seemingly independent of the other. Elevated amino acids include alanine, glutamine, glutamate, lysine, and alpha-amino-N-butyrate. Amino acids that are normal or slightly increased include ornithine, aspartate, arginine, tyrosine, phenylalanine, methionine, leucine, isoleucine, valine, taurine, threonine, serine, proline, glycine, and histidine. Citrulline and argininosuccinic acid are not de-

tectable. Amino acids usually not found in the serum also may be detected including cystathionine, homocystine, beta-aminoisobutyric acid, and saccharopine.^{42,44,54,59} Elevated organic acid concentrations include lactic, pyruvic, beta-hydroxybutyric, acetoacetic, butyric, isobutyric, propionic, isovaleric, and caprylic.^{42,111,113} The fatty acid patterns of serum free fatty acids, triglycerides, and phospholipids are distinctly different in patients with Reye syndrome. The polyunsaturated fatty acid content of phospholipids is decreased and the polyunsaturated fatty acid content of the free fatty acid fraction is increased.⁷⁵ Ogburn and coworkers have postulated that the antecedent viral infection releases phospholipase A₂, which releases polyunsaturated fatty acids from tissue phospholipids into the free fatty acid pool, thereby stimulating prostaglandin synthesis. This metabolic cascade, in turn, leads to dysfunction of the liver and brain. The serum cholesterol concentration is decreased. Also, several serum proteins are decreased in amount or activity including lipoproteins (VLDL), clotting factors,^{80,95} and components of the complement system.^{66,81}

The concentrations of several circulating hormones also have been measured in Reye syndrome.^{42,73} Plasma insulin concentrations are appropriate for the blood glucose concentrations. Plasma cortisol concentrations are markedly elevated; plasma glucagon, growth hormone, and prolactin concentrations are less strikingly elevated. Also, the plasma concentrations of cyclic adenosine monophosphate (AMP) have been found to be elevated,⁵³ and aberrant activity of hepatic cyclic nucleotide phosphodiesterase has been reported.⁵²

The decrease in the serum VLDL concentration and the absence of lipoprotein particles in the Golgi membranes observed by electron microscopy in liver biopsies suggests that a transient impairment in the extrahepatic transport of intracellular lipid exists in this disease process.⁷⁷ Pollack and coworkers have speculated that the antecedent viral illness facilitates fatty acid mobilization from peripheral adipose tissues, resulting in excessive lipolysis and free fatty acidemia.⁸³ This speculation is consistent with the more recent observations by Ogburn and coworkers.⁷⁵

The histopathology of Reye syndrome has been studied in considerable detail. Changes in the liver include microvesicular steatosis, glycogen depletion, depleted Golgi membranes, proliferation of peroxisomes, and distorted mitochondria.⁷⁷ Mitochondrial abnormalities similar to those described in the hepatocyte have been noted in neurons, together with a peculiar unraveling and bleb formation of myelin, reminiscent of the myelin disturbances produced by the toxins hexachlorophene and triethyltin. Qualitatively similar abnormalities of mitochondria together with lipid droplet accumulation and glycogen depletion have been noted in muscle biopsies.^{41,96} Type I muscle fibers are particularly vulnerable to these histologic changes.

The biochemical studies are consistent with the histopathologic observations; namely, that there is a universal decrease in the activity of all mitochondrial enzymes measured to date, with the sole excep-

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tion of carnitine palmitoyltransferase.¹¹⁶ The mitochondrial enzymes that are decreased in activity include ornithine transcarbamylase, carbamoyl-phosphate synthetase, the pyruvate dehydrogenase complex, pyruvate carboxylase, succinate dehydrogenase, cytochrome oxidase, glutamate dehydrogenase, isocitrate dehydrogenase, and monoamine oxidase.^{25,69} Whether other mitochondrial enzyme systems are involved in this disease process is unclear, as a complete analysis of these enzymes has not been undertaken. By contrast, the cytosolic enzymes that have been measured have normal activities.^{38,69} Resolution of the malate dehydrogenase isoenzymes by electrophoresis demonstrated that the cytosolic-to-mitochondrial isozyme ratio was significantly increased in patients with Reye syndrome.⁶⁹ Decreased activity of the mitochondrial isozyme accounted for the elevated ratio.

The histopathologic and biochemical observations have permitted investigators to speculate that Reye syndrome represents a metabolic response to a universal mitochondrial insult.²⁵ Most, if not all, of the laboratory abnormalities may be explained on the basis of a primary mitochondrial injury.¹¹

EPIDEMIOLOGY

Certain facts are well established as they relate to the epidemiology of this illness. There is a specific relationship between two viruses, varicella and influenza, and Reye syndrome. Approximately 25 per cent of patients with Reye syndrome have varicella as the antecedent illness, and most of the remaining patients have influenza B or A as the antecedent infection. Other viral illnesses have been implicated, but less well documented, and occasional reports describe serologic evidence of simultaneous viral infections. The attack rate for Reye syndrome approximates 30 to 60 cases per 100,000 influenza B infections, 2.5 to 4.3 cases per 100,000 influenza A infections, and 0.3 to 0.4 cases per 100,000 varicella infections.² The temporal relationship between the seasonal occurrence of influenza B and epidemics of Reye syndrome has been documented on several occasions.^{15,70,107} The sporadic cases are distributed more evenly throughout the year and usually follow varicella. Various epidemiologic surveys have shown that Reye syndrome is predominantly a rural-suburban entity rather than an urban entity. The urban-rural distribution of Reye syndrome in Ohio reported by Sullivan-Bolyai and coworkers¹⁰⁷ differed from the previous reports because the demographic classifications were redefined according to the 1970 U.S. Bureau of Census Definitions. The urban-noncentral city standard metropolitan statistical area demonstrated the highest rate (1.62 cases per year per 100,000 persons age 0 to 17 years). This study also suggested that rates of attack were lower in the low socioeconomic groups. These collective observations suggested that Reye syndrome may be influenced by individual susceptibility or the micro environ-

ment, rather than the global environment; that is, within and around the household as opposed to industrial or farming considerations. Lichtenstein and coworkers,⁶⁰ using only biopsy-proven cases, determined an even higher incidence of 3.5 cases per 100,000 children under the age of 17 years in metropolitan Cincinnati during a 1-year prospective study. This incidence figure was 11-fold higher than the incidence calculated by the Centers for Disease Control for the same time period. Lichtenstein and associates excluded some patients as examples of Reye syndrome because the histochemical stain for succinic dehydrogenase was equivocal or normal in the liver biopsy specimens. Inclusion of these patients gives a higher incidence figure approaching 6.06 cases per 100,000 children less than 1 to 17 years of age.²⁴

These epidemiologic surveys have suggested the possibility that environmental factors may be contributory in Reye syndrome acting synergistically with an antecedent viral infection. A number of hypotheses have been advanced regarding insecticides, insecticide carriers, and emulsifiers as possible environmental toxins, but no confirmatory data have been forthcoming.²⁰ In addition, aflatoxins and salicylates have been implicated. For the moment, however, there is no compelling evidence linking any of these environmental toxins causally with Reye syndrome.

ETIOPATHOGENESIS

The abundance of histologic and metabolic abnormalities documented in Reye syndrome have tantalized students of this malady for years. Despite these clues, the etiopathogenesis remains obscure. The clinical phenotype has been mimicked by environmental intoxicants and various inborn errors of metabolism. Intoxication with salicylates, tetracyclines, valproic acid, disulfiram, phenformin, margosa oil, chlordane, pyrrolizidine, camphor, methylbromides, and lead produce a similar clinical syndrome.^{33,50,76,90,97,101,103,106}

Several attempts have been made to identify a genetic predisposition in Reye syndrome, without success. The syndrome rarely recurs in patients.^{36,93,114} Sibling involvement in Reye syndrome has been documented on several occasions.^{15,24,90} Siblings of either sex and twins have been reported to be affected, and the antecedent infection often was varicella or influenza B. One sibling also had a diagnosis of ketotic hypoglycemia. Hilty⁴³ reported three families with sibling involvement. Varicella was the antecedent infection in two families and an upper respiratory tract infection in the third. Wilson¹¹⁷ reported the simultaneous occurrence of Reye syndrome in three siblings with serologic data indicating that each had an H1N1 influenza virus infection. There was no evidence of haplo-type segregation by HLA typing in either report. Similarly, neither Hilty nor Wilson was able to identify any environmental factor contributing to the simultaneous occurrence of disease in the siblings.

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Initially, it was thought that the high mortality precluded recurrences. However, this explanation has become less tenable in recent years as the apparent mortality rate has declined. Also, more patients with "recurrent Reye syndrome" have been shown now to have inborn errors of metabolism. Ornithine transcarbamoylase deficiency, systemic carnitine deficiency, medium-chain and long-chain acyl-CoA dehydrogenase deficiencies, beta-hydroxy-beta-methylglutaric acidemia, isovaleric acidemia, and the various ketotic hyperglycinemic syndromes may demonstrate the Reye syndrome phenotype.^{14,35,39,55,59,87,100,104} The recurrent nature of these inherited disorders is an important distinction. Recurrent attacks of "Reye syndrome" strongly imply an inborn error of metabolism unless proved otherwise. The laboratory abnormalities differ among these various disorders although varying degrees of hepatic dysfunction may be shared by all these entities. The absence of ketonuria is characteristic of systemic carnitine deficiency, medium-chain acyl-CoA dehydrogenase deficiency, and beta-hydroxy-beta-methylglutaric acidemia. Elevated ketone body concentrations occur in isovaleric acidemia, propionic acidemia, and methylmalonic aciduria. Increased urinary excretion of orotic acid, particularly between acute episodes, is characteristic of ornithine transcarbamoylase deficiency.

The pathophysiology of the clinical state may be similar in Reye syndrome and the other conditions that present with a similar phenotype. Several observations suggest that a child suffering from Reye syndrome is subjected to intense metabolic stress. There is tissue glycogen depletion, excessive peripheral lipolysis, and impaired biosynthetic processes. Disturbed protein synthesis is suggested by decreased pre-beta-lipoproteins, several clotting factors, and components of the complement system.⁶⁶ However, it remains unclear which of these metabolic abnormalities is primary and which is secondary. The relationship between the etiologic event, namely the viral infection and the subsequent mitochondrial injury, remains elusive; but there is circumstantial evidence to suggest an intracellular impairment in various biosynthetic and storage processes. Mitchell and coworkers⁶⁹ extended this speculation by suggesting that Reye syndrome may reflect a generalized decrease in the steady state level of mitochondrial enzymes because of selective impairment of mitochondrial biogenesis.

The mechanism of the encephalopathy also remains controversial.²² Some consider it to be the consequence of hepatic failure, whereas others believe it is a manifestation of the primary mitochondrial injury suffered by many organs including liver and brain. Elevated blood ammonia concentrations were implicated initially as the possible cause of the encephalopathy.⁷⁴ More recently, investigators have focused on the free fatty acidemia as the pathogenetic mechanism.^{3,10,111-113} Animal models have been developed to explore this mechanism further by infusing sodium octanoate.¹¹² These experiments also represent a model for the inherited deficiency of medium-chain acyl-CoA dehydrogenase, which simulates Reye syndrome.

A previous interest in salicylates as a pathogenetic factor has been rekindled by epidemiologic studies that document a statistical association between salicylate intake and the development of Reye syndrome.^{40,79,80} These studies have been criticized because of methodologic flaws. Nevertheless, the current prevailing bias is that salicylates represent an added metabolic insult to an organism already burdened by a primary mitochondrial injury.

The apparent similarities between salicylate intoxication and Reye syndrome have intrigued investigators for the past decade. Hepatic dysfunction and encephalopathy occur in both settings, although hyperammonemia is never striking in salicylate intoxication.^{9,29,71,90} Other similarities include a mixed acid-base disturbance and a coagulopathy.⁴ However, important differences exist including the hepatic histopathology and the nature of the amino acidemia.^{8,69} The reported serum salicylate concentrations in patients with Reye syndrome often fall within the therapeutic range.⁷⁹ However, these salicylate concentrations may be inappropriately high given the interval of time between the last dose of aspirin and the measurement of the serum concentration. One brief study documented a prolonged biologic half-life for aspirin during Reye syndrome;⁸⁸ another has reported decreased aspirin esterase activity.¹⁰⁹ These abnormalities disappeared after recovery. Salicylate also can produce gross swelling of isolated mitochondria suspended in isotonic salt solutions.¹¹⁹ Given our current understanding of the problem, it seems prudent to conclude that a statistical association exists between salicylate intake and Reye syndrome. It is likely that this observation represents a compounding of the pathophysiology of Reye syndrome rather than a causal relationship. Other reports have indicated that Reye syndrome has developed in children who have taken acetaminophen rather than aspirin. Poisoning by acetaminophen causes centrilobular hepatic necrosis, and the toxic effects of this substance can be potentiated in mice by concurrent infection with influenza B virus.⁶⁵ Hepatic necrosis occurs infrequently in Reye syndrome; when present, it is usually periportal in distribution.⁵ This pattern of distribution has suggested a toxic insult to the liver as may be seen with high concentrations of fatty acids or aflatoxin B.¹¹ The periportal distribution in Reye syndrome differs from the centrilobular pattern associated with acetaminophen intoxication.

Aflatoxin is a metabolite of the fungus *Aspergillus flavus*. This toxin has been suggested as an etiologic agent in Reye syndrome and was first identified in children with Udorn encephalopathy, an illness that resembles Reye syndrome.⁶ Recent studies have shown that aflatoxin selectively depresses the mitochondrial enzymes carbamyl-phosphate synthetase and ornithine transcarbamoylase without affecting the cytosolic urea cycle enzyme arginase.¹⁰⁶ One study has demonstrated aflatoxin B₁ in 11 of 14 liver samples, 4 of 6 blood samples, and 4 of 5 urine samples obtained from patients with Reye syndrome.¹⁰² A second study, however, has failed to demonstrate any significant difference in aflatoxin levels in serum and urine obtained

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Aspergillus flavus. This Reye syndrome and encephalopathy, an illness have shown that aminotransferases carbamyltransferase without aflatoxin.¹⁰⁶ One study has shown that 4 of 6 blood samples from patients with Reye syndrome demonstrate abnormalities and urine obtained

from patients with Reye syndrome when compared with control subjects.¹² Twenty-three per cent of all patients in this second study had measurable aflatoxin concentrations indicative of recent exposure. This observation suggests that people in the United States are constantly exposed to low levels of aflatoxin, a finding that may have public health significance.

Other xenobiotic initiating factors have been looked for in patients with Reye syndrome because of the epidemiologic data and the known association of enhanced viral virulence with certain chemicals in animals, insects, and cell cultures.⁸² Environmental toxins such as aflatoxins, herbicides, insecticides, and surfactants have been studied in various animal models.⁴⁷ The ferret model also has been exploited, because this animal is susceptible to the human influenza virus.^{23,61} Many of the histopathologic and biochemical abnormalities found in Reye syndrome have been replicated in these diverse animal models. The primary flaw, unfortunately, of these model systems is the lack of confirmatory evidence of a xenobiotic initiating factor in the tissues or biologic fluids of children with Reye syndrome. Davis and colleagues recently reported on their studies of experimental influenza B virus toxicity in mice.²¹ Juvenile BALB/c mice were injected intravenously with a nonmouse-adapted strain of influenza B/Lee/40 virus. Lethargy, seizures, coma, and death developed 1 to 3 days later. The clinical, biochemical, and pathologic features of the mouse illness were remarkably similar to the human illness. The serum transaminases and plasma ammonia were elevated, the brain was swollen without any inflammatory changes, and the liver demonstrated microvesicular fatty metamorphosis. Viral propagation did not occur in brain or liver. Ultrastructural examination of the liver revealed microvesicular steatosis, mild glycogen depletion, and slight matrix expansion of occasional mitochondria. No viral particles were seen. Previous studies by these investigators also documented biochemical abnormalities in the livers of these animals. A particularly interesting observation is the abnormal compartmental redistribution of ornithine transcarbamoylase to the cytoplasm.¹¹⁷ This enzyme is encoded by the nuclear genome, and the gene product then enters the mitochondrial matrix to assume its catalytic function in the urea cycle. Brownstein and colleagues observed a spontaneous viral illness occurring in BALB/cByJ mice that included the cardinal clinical and laboratory features of the human counterpart.¹² The mice developed histopathologic and chemical evidence of liver disease and clinical and histopathologic evidence of an encephalopathy, with convincing ultrastructural evidence of prominent mitochondrial injury. Unlike the occasional findings in the Davis model, the mitochondrial findings were striking both in liver and brain and very similar to the findings in Reye syndrome. Absence of very low density lipoprotein from the Golgi complexes was also noted. The prominence of cerebral astrocytosis was unusual and more reminiscent of a chronic hepatic encephalopathy. The mouse syndrome appears to have followed a coronavirus intestinal infection in 66 per cent of the cases. This report

by Brownstein represents the first documentation of a spontaneous illness resembling Reye syndrome developing in an animal. This model and the Davis model may prove to be important in studying the early phase of Reye syndrome, which currently seems unapproachable in the human setting.

TREATMENT

Early diagnosis is crucial to satisfactory outcome. Most patients present a stereotyped clinical and laboratory profile. The diagnosis is obvious in most situations, particularly if the physician has had previous experience with the syndrome. The diagnostic criteria include the following clinical and laboratory elements.

1. The presence of an antecedent viral illness
2. A latent interval of several days before the onset of pernicious vomiting
3. The development of a diffuse encephalopathy
4. No other obvious explanation for the encephalopathy
5. A three-fold or greater elevation of the serum transaminase activities
6. Prolongation of the prothrombin time
7. Hyperammonemia
8. Normal CSF examination.

Occasionally, the opening pressure at the time of lumbar puncture may be elevated and hypoglycorrhachia may be present in association with hypoglycemia. The advisability of a lumbar puncture has been challenged.⁶⁸ It has been our practice to perform a lumbar puncture as part of the initial evaluation, particularly if the diagnosis is in doubt. The presence in the CSF of white or red blood cells or the elevation of the protein concentration should cast doubt on the diagnosis and other possibilities should be considered. It is our belief that a lumbar puncture performed later in the course of treatment may be hazardous, with an increased likelihood of central herniation. Therefore, the lumbar puncture should not be performed later unless there is a compelling reason, for example, increasing concern about the possibility of intracranial infection.

Over the past 15 years we have developed a standardized form of intensive medical support for patients with Reye syndrome.^{26,42} The management of the individual patient is determined by the neurologic "stage" of the child at the time of admission to hospital. We define stage I as a mildly affected patient with subtle behavioral disturbances such as inattention, inappropriateness, lethargy, somnolence, confusion, or mild irritability. Stage II describes the sicker child who is disoriented or demonstrates agitated delirium, stupor, or coma associated with decorticate posturing of the limbs and trunk. Overactivity of the sympathetic nervous system is manifested in stage II by tachycardia, systemic hypertension, hyperthermia, hyperpnea, diaphoresis, and dilated pupils. Stage III describes the gravely ill and comatose child with decerebrate posturing of limbs and trunk. The

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eyes are intermittently or continuously forced into down gaze, indicative of midbrain dysfunction. Stage IV describes the moribund state with loss of brain-stem function. Pupils are nonreactive, spontaneous breathing has ceased, pulse is rapid and weak, blood pressure is low, and pulmonary congestion is present.

All patients with Reye syndrome should be hospitalized immediately. It is now generally accepted that early vigorous treatment will limit the progression of the syndrome.⁹⁴ Children in stage I are managed by intravenous hydration with a 10 per cent hypertonic glucose-multielectrolyte solution. Intravenous fluids are administered at a rate of 1600 to 1800 ml per m² per day. Vitamin K (Aquamephyton) is administered every 24 hours at a dose of 1 mg intravenously or 5 mg intramuscularly. Approximately 90 per cent of children admitted to the hospital in stage I will remain stable and make an uneventful recovery. The remaining 10 per cent of patients will deteriorate neurologically and require more intensive medical support.

Children in stages II through IV and those children who have progressed to stage II after being admitted in stage I should be transferred to the intensive care unit. A servocontrolled cooling blanket should be placed on the bed, and a number of procedures should be carried out while the patient is anesthetized and paralyzed with 4 mg per kg of sodium thiopental (Pentothal) intravenously and 1 mg per kg of succinylcholine (Anectine) intravenously. These medications should be repeated as necessary to permit completion of the procedures. During this time, the patient should be hyperventilated, maintaining an arterial carbon dioxide tension of approximately 25 mm Hg. The procedures include placement of a nasotracheal tube, radial artery catheter, central venous catheter, nasogastric tube, and urinary catheter. These procedures should be carried out as expeditiously as possible by experienced personnel. We prefer to thread the central venous catheter through the superficial saphenous vein into the inferior vena cava for delivery of hypertonic solutions of glucose and mannitol. Occasionally, access to the subclavian vein necessitates placing the child in the Trendelenburg position; this position may aggravate the coexisting intracranial hypertension. The head is turned to one side to carry out this procedure; this position may compromise venous drainage of the head. Also, there is a tendency to drape the patient's head and neck when access to the subclavian vein is being attempted, thereby precluding minute-to-minute observation of the patient during this critical time. A femoral approach obviates these concerns. The central venous catheter permits continuous infusion of the hypertonic glucose solution and intermittent administration of hypertonic mannitol. The gastric contents should be emptied through the nasogastric tube, and the tube should then be placed to intermittent low suction to prevent subsequent regurgitation. The lumbar puncture, if it is to be performed, should be carried out at this time, preferably while the patient remains sedated and paralyzed with thiopental and succinylcholine.

The liver biopsy is reserved for the more puzzling atypical cases, for "recurrent" cases, and for infants. The coagulopathy must be corrected before this procedure is performed.

The Reye syndrome solution, which is administered to patients in stages II through IV, contains 200 gm of glucose, 40 mEq of sodium chloride, 15 mEq of potassium acetate, and 15 mEq of potassium phosphate per liter. One ampule (10 cc) of multiple vitamins (MVI) is added per liter of solution, and this solution is infused at a daily rate of 1600 to 1800 ml per m^2 . This daily rate of infusion provides approximately 500 mg glucose per kg body weight per hour and an adequate fluid volume to permit gradual rehydration of the dehydrated patient. The blood glucose concentration usually reaches 250 to 350 mg per dl. Lower blood glucose concentrations are seen in patients who are less compromised metabolically; no further effort need be made to achieve higher circulating glucose concentrations. Blood glucose concentrations in excess of 400 mg per dl may occur in the more desperately ill patients who are severely compromised metabolically. The glucose concentration of the Reye syndrome solution may be decreased to 15 per cent or 10 per cent as necessary under such circumstances, while still maintaining a constant fluid rate of 1600 to 1800 ml per m^2 per day. Maintaining glucose concentrations in the range of 300 mg per dl lessens the requirement for administration of mannitol and provides more optimal circulating concentrations of glucose to meet increased apparent rates of cerebral glycolysis. We have assumed, but it has not been proved, that the brain glucose requirements are relatively increased in Reye syndrome during the time that mitochondrial mechanisms are compromised. We also believe that it is important to maintain a constant rate of glucose infusion once the patient has adapted metabolically and has stabilized after the initial diagnostic and therapeutic procedures have been completed. Continual readjustments of the fluid rate should be discouraged. Continual fluctuations of the glucose concentration appear to be associated with continuing clinical and metabolic instability of the patient. The constant infusion of glucose should never be interrupted; otherwise, the blood glucose concentration and the associated serum osmolality will decrease rapidly.

Intracranial hypertension is managed primarily by the administration of 20 per cent hypertonic mannitol. We are now inclined toward earlier placement of a Ladd epidural monitor to determine the need for mannitol. All patients in stage III, and more patients in stage II, have an epidural monitor placed through a burr hole overlying the right frontal cortex. The procedure is carried out by a neurosurgeon in the intensive care unit using local anesthesia. Thiopental and succinylcholine may be administered as necessary to facilitate this procedure. Intravenous boluses of hypertonic mannitol (0.25 gm per kg) often are sufficient to control the intracranial hypertension. The dose of mannitol is administered ("piggy-backed") through the central venous catheter over 3 to 5 minutes. This dosage of mannitol may be repeated as frequently as necessary. Alternatively, larger

doses of mannitol are administered. Large doses (10 to 30 mEq per kg) of blood pressure support. In cases usually are not sufficient, and often are followed by a decrease in blood pressure. As a result, a more aggressive regimen of fluid administration per liter, and a higher osmolarity (mOsm per kg) and mannitol also are used for drainage.

We have found it possible, in many cases, to maintain values of blood glucose. Most patients with a T-tube for drainage assistance, an altered level of consciousness. Elective intensive intervention is carried out after the patient is medicated, and the dramatic effect of the

Few patients develop hypothermia below normal body temperature of blood, and a cent of the hemorrhage in the whole blood.

Patients with hypotension, tachycardia, and every effort is made to approach normal values, provide mechanical ventilation, sedation, and a living system.

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doses of mannitol ranging from 0.5 to 2.0 gm per kg may be administered. Larger doses should be infused over longer periods of time (10 to 30 minutes) to minimize the transient elevations of systemic blood pressure associated with expansion of the vascular compartment. In our experience, daily mannitol doses of 4 to 6 gm per kg usually are sufficient to control cerebral edema. Smaller daily doses often are adequate. We believe that the induced hyperglycemia resulting from the infusion of a 20 per cent glucose-containing solution decreases the daily requirement of mannitol in these patients. As a result, malignant hyperosmolality is seldom encountered with this regimen. Rarely do we encounter osmolalities in excess of 320 mOsm per L, and most patients maintain a serum osmolality of 290 to 310 mOsm per L. Elevation of the head of the bed by 20 to 30 degrees and maintenance of the head and neck in a neutral midline position also are important considerations to facilitate cerebral venous drainage.

We prefer to allow the patient to breathe independently, if possible, maintaining a state of mild hyperoxia and hypocapnia with P_{aO_2} values of 100 to 150 mm Hg and P_{aCO_2} values of 20 to 27 mm Hg.²⁷ Most patients in stage II and early stage III maintain these arterial values spontaneously while breathing humidified oxygen through a T-tube adapted to the nasotracheal tube. Alternatively, ventilatory assistance in the IMV mode is necessary, particularly if a patient has an altered respiratory pattern, hypoventilation or periodic breathing. Elective nasotracheal intubation immediately upon admission to the intensive care unit ensures control of the patient's airway and permits intervention when necessary. Suctioning of the airway should be carried out carefully and coordinated with administration of mannitol or after the administration of thiopental and succinylcholine, when these medications are indicated for other procedures. Excessive suctioning of the patient under other circumstances often is associated with dramatic elevations of the intracranial pressure.

Fever is managed by the use of a cooling blanket and acetaminophen suppositories. No effort is made to lower the body temperature below normal. The gastric contents commonly contain small amounts of blood, but no specific treatment is necessary. Less than 10 per cent of patients with Reye syndrome develop clinically significant hemorrhaging requiring the infusion of freshly frozen plasma or fresh whole blood.

Pentobarbital may be administered to patients with intracranial hypertension that persists despite frequent doses of mannitol.⁶⁷ Pentobarbital doses of 1 to 5 mg per kg intravenously may be repeated every 4 to 8 hours as necessary to achieve a serum concentration of approximately 30 to 50 mg per L. These doses often are adequate to provide control of the intracranial hypertension. Increasing use of mechanical ventilation becomes necessary as the patient is further sedated by the administration of pentobarbital. Complications deriving from pentobarbital include impaired cardiac output and falling systemic blood pressure.³⁴ Systemic hypotension may be more de-

vastating than intracranial hypertension, particularly as it relates to cerebral metabolic activity. Accordingly, we recommend the judicious use of pentobarbital in the lowest dose possible to achieve adequate control of the intracranial hypertension. In most cases mannitol is adequate for control of intracranial hypertension, and pentobarbital is not necessary. Seizures, when they occur in gravely ill patients, also may be managed with pentobarbital. Phenobarbital may be used instead of pentobarbital, but the longer-acting barbiturate has no particular therapeutic advantage.

The therapeutic goal in the management of patients with Reye syndrome is to achieve a metabolic steady state as soon as possible after the initial diagnostic and therapeutic procedures have been carried out. Relatively few adjustments are necessary once the patient has adapted metabolically to the intravenous fluids and to the respiratory settings. It is important to minimize stimuli that arouse the patient, as dramatic increases of intracranial pressure may occur. Repeated neurologic examinations by multiple observers is particularly inappropriate under these circumstances. Careful serial observations of the patient's posturing, size and reactivity of the pupils, and positioning of the eyes provide sufficient information to gauge the clinical course. This information, together with the serial laboratory observations, is sufficient to monitor the patient's progress.

A battery of blood studies are obtained routinely at the time of the patient's admission to the hospital. These studies include a total hematologic and chemical profile, coagulation studies, ammonia, osmolality, and lactate concentrations. These studies are repeated every 24 hours. In addition, the blood concentrations of glucose, lactate, osmolality, pH, P_{aO_2} , and P_{aCO_2} are obtained every 4 hours. These studies are sufficient to monitor the metabolic progress of the patient. Hypophosphatemia commonly is present, and the serum phosphorus concentration would decline further after administration of hypertonic glucose.^{17,56} It is important, therefore, to maintain adequate amounts of phosphate in the Reye syndrome solution to buffer this tendency. A falling serum calcium concentration may be an indication of pancreatic involvement.³⁷ This complication may be devastating; fortunately, it is rare and usually associated with the administration of corticosteroids.^{15,31} There is little or no indication for the use of corticosteroids in the management of patients with Reye syndrome.

Most patients, treated in this manner demonstrate clinical and laboratory improvement within 24 to 72 hours. Once consciousness has been regained, the glucose concentration and the Reye syndrome solution is decreased by 25 per cent decrements every 8 hours and the patient is extubated. Usually within 24 hours of regaining consciousness, patients are able to take liquids by mouth, and the remaining catheters may be removed.

Our experience with this current regimen or one of its earlier versions involves 61 patients from 1971 to 1983. Fifty-four patients have survived, three have had residual neurologic deficits, but in each case they have returned to school. The patients are summarized in Table 1.

REYE SYNDROME

Table 1. Re

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Table 1. Reye Syndrome Treatment Protocol: Results (1971-1983)

ADMISSION STAGE	NO. OF PATIENTS	OUTCOME	
		Alice	Dead
I	13	13	0
II	33	33	0
III	15	8	7
IV	0	0	0
Total	61	54 (89%)	7 (11%)

ALTERNATIVE THERAPEUTIC CONSIDERATIONS

The medical management of patients with Reye syndrome has become increasingly standardized over the past decade, thereby minimizing alternative considerations. Generally accepted as standard treatment is the use of hypertonic glucose and hypertonic mannitol, early elective intubation, placement of the patient in an intensive care unit setting if they deteriorate beyond grade I, parenteral administration of vitamin K and monitoring of the intracranial pressure.^{74,92,110} Various techniques have been used to monitor the intracranial pressure including placement of the sensor either in the epidural space, subarachnoid space, or the lateral ventricle.⁹⁸ Each technique has its advantages and disadvantages. On balance, we have been quite satisfied with the use of a Ladd epidural monitor and a similar experience has been reported from Cincinnati.³³ Controversies still remain about the advisability of exchange transfusions,^{18,70} deep hypothermia, barbiturate-induced coma,^{34,67} and bilateral craniectomies for the relief of intractable cerebral edema.⁷⁸ Other alternatives have been discarded including peritoneal dialysis and total body asanguinous perfusion.⁵⁹ Available statistics, uncontrolled as they are, clearly suggest that intensive medical support of the patient with Reye syndrome produces satisfactory results as often as, or more frequently than, any other approach.^{18,70,92}

Current evidence suggests that corticosteroid therapy may be associated with a higher mortality and may contribute to pancreatic complications. It continues to be our impression that the frequency of complications in Reye syndrome is determined, in part, by the choice of management. Restriction of fluid, together with administration of hyperosmolar agents clearly contributes to prerenal azotemia and renal failure. Pulmonary complications appear to be more common in patients subjected to deep hypothermia and perhaps also to barbiturate-induced coma. Barbiturates also increase the possibility of cardiovascular collapse with attendant hypoperfusion of the brain.

Fortunately, considerable progress has been made in the care of patients with Reye syndrome. Today, most patients make a full and complete recovery without any neurologic or psychological sequelae.⁹⁹ The sequelae associated with Reye syndrome probably derive in large part from attendant complications that occur during the

acute encephalopathy including hypoglycemia, systemic hypotension with resulting cerebral ischemia, hypoxia, and uncontrolled intracranial hypertension. The therapeutic approaches now available minimize the likelihood of these complications and maximize the quality of recovery.

SUMMARY

Reye syndrome has emerged as the quintessential example of an acute metabolic encephalopathy. The clinical presentation is quite stereotyped in most instances permitting rapid, accurate diagnosis and early therapeutic intervention. Intoxications and certain inborn metabolic errors may mimic Reye syndrome. All patients with a recurrent episode should be investigated thoroughly for evidence of a metabolic disorder associated with an enzyme deficiency. Notable in this regard are inborn errors affecting organic acid, ammonia, and carbohydrate metabolism. The mitochondrial disturbance in Reye syndrome is well documented but the pathophysiologic sequence linking the antecedent viral illness to the mitochondrial injury remains obscure. Recent identification of a spontaneous Reye-like illness in mice that is associated with a coronavirus infection may provide an opportunity to investigate this initial phase of the pathophysiologic sequence.

The primary cerebral insult presumably derives from insufficient substrate availability and results in massive cytotoxic cerebral edema. Treatment revolves around the continuous infusion of hypertonic glucose and intermittent infusion of hypertonic mannitol. Management is designed to attenuate or avoid the various compounding metabolic insults during this critical period when the child is metabolically crippled. In 1963, the disorder was considered to be rare and almost irreversibly fatal. Today, the disorder is recognized to be more common, and the outcome is very satisfactory in 85 to 90 per cent of the cases. The role of aspirin remains very controversial. A number of studies suggest an association between this potential mitochondrial toxin and Reye syndrome, but a causal relationship has not been established. Until better understood, it seems advisable to avoid use of aspirin in children exhibiting symptoms suggestive of Reye syndrome.

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REYE SYNDROME

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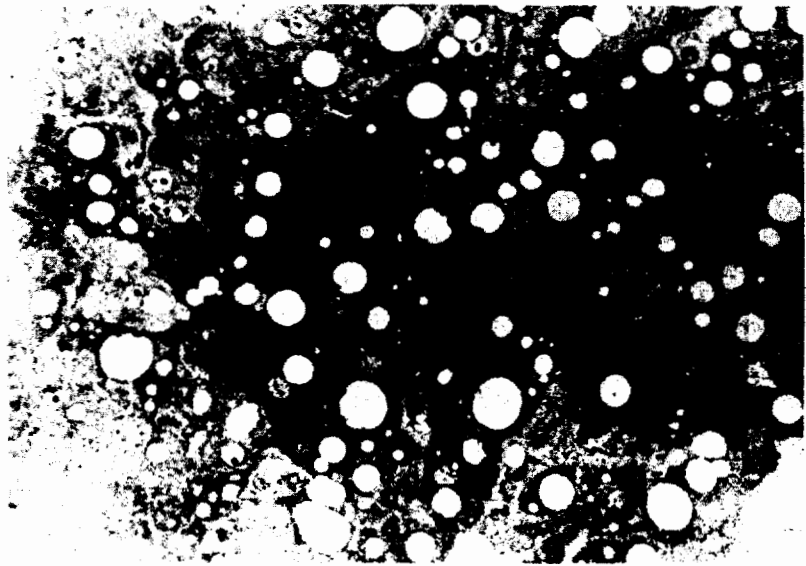
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REYE'S SYNDROME

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DORIS A. TRAUNER, M.D.

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SELF-ASSESSMENT QUESTIONS

1. Which of the following has the most frequent development of Reye's syndrome?
 - a. Live virus vaccines.
 - b. Epstein-Barr virus.
 - c. Herpes simplex.
 - d. None of the above.
2. Which of the following pathologic changes is characteristic of Reye's syndrome?
 - a. Periportal inflammation.
 - b. Microvesicular fatty accumulation.
 - c. Hepatocellular necrosis.
 - d. All of the above.
3. Which of the following is true of Reye's syndrome?
 - a. Girls are affected more often than boys.
 - b. Children over 1 year of age are affected.
 - c. Infants under one year of age are affected.
 - d. All of the above.
4. Biochemical abnormalities that may be found include all of the following except:
 - a. Hyperammonemia.
 - b. Lactic acidemia.
 - c. Hyperbilirubinemia.
 - d. Prolonged prothrombin time.
5. The diagnosis of Reye's syndrome requires all of the following criteria?
 - a. Clinical progression of illness, including vomiting, behavioral changes, and coma.
 - b. Laboratory evidence of liver dysfunction.
 - c. Exclusion of CNS infection by appropriate tests.
 - d. All of the above.
6. Indications for liver biopsy include the following:
 - a. All children with suspected liver disease.
 - b. All children over 1 year of age.
 - c. Infants under 1 year of age.
 - d. None of the above.

SELF-ASSESSMENT QUESTIONS

1. Which of the following has *not* been associated with subsequent development of Reye's syndrome?
 - a. Live virus vaccines.
 - b. Epstein-Barr virus.
 - c. Herpes simplex.
 - d. None of the above.
2. Which of the following pathologic features is characteristic of Reye's syndrome?
 - a. Periportal inflammation.
 - b. Microvesicular fatty accumulation.
 - c. Hepatocellular necrosis.
 - d. All of the above.
3. Which of the following is true concerning the epidemiology of Reye's syndrome?
 - a. Girls are affected more commonly than boys.
 - b. Children over 1 year of age are typically white, middle-class and suburban dwellers.
 - c. Infants under one year of age are typically white, middle-class and suburban dwellers.
 - d. All of the above.
4. Biochemical abnormalities found in Reye's syndrome include all of the following except:
 - a. Hyperammonemia.
 - b. Lactic acidemia.
 - c. Hyperbilirubinemia.
 - d. Prolonged prothrombin time.
5. The diagnosis of Reye's syndrome is made on which of the following criteria?
 - a. Clinical progression of prodromal viral illness to vomiting, behavioral changes or coma.
 - b. Laboratory evidence of hepatic dysfunction.
 - c. Exclusion of CNS infection and toxin ingestion by appropriate tests.
 - d. All of the above.
6. Indications for liver biopsy in suspected Reye's syndrome include the following:
 - a. All children with suspected Reye's syndrome should have a liver biopsy.
 - b. All children over 1 year of age.
 - c. Infants under 1 year of age.
 - d. None of the above.

7. The differential diagnosis of Reye's syndrome includes the following:
 - a. Varicella hepatitis/encephalitis.
 - b. Salicylate ingestion.
 - c. Anoxic encephalopathy.
 - d. All of the above.
8. Which of the following exogenous toxins has been associated with Reye's-like illnesses in humans?
 - a. Uric acid.
 - b. Aflatoxin B1.
 - c. Methionine.
 - d. None of the above.
9. Treatment of Reye's syndrome consists of the following:
 - a. Hypertonic glucose.
 - b. Neomycin by nasogastric tube or enema.
 - c. Controlled hyperventilation.
 - d. All of the above.
10. Which of the following is true concerning the prognosis for Reye's syndrome?
 - a. Mortality is currently 80-100%.
 - b. Most children who survive have severe neurologic damage.
 - c. Most children over the age of 2 years who survive return to grossly normal functioning.
 - d. Survival rates have not changed significantly over the past 15 years.

Answers are listed at the end of the article.

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HISTORICAL BACKGROUND

THE SYNDROME of "encephalopathy of the viscera" was first described in Australia by Reye, Morgan, and colleagues in 1963. It affects 20 children ranging in age from 1 to 12 years who developed a viral illness, fever, convulsions, vomiting, and coma. Many of the patients had aminases and hypoglycemia with fatty acid accumulation in the liver. The brains showed evidence of cerebral edema and diffuse fatty accumulation in the liver.

Although Reye has been credited with the description of this disorder, a review of the literature shows that it is not new. An association between the development of "toxic" encephalopathy and fatty acid accumulation in the liver was first reported by Reye in 1895. In 1929, Brain et al. described an encephalitis-like illness who had fatty acid accumulation in the liver. In 1961, Reye et al. described encephalopathies of obscure origin in which virtually all the cases they presented had antecedent viral illnesses who developed respiratory alterations (usually respiratory arrest), and seizures. The serum transaminases were not elevated and were confined to the brain, while anoxic changes were observed. The disorder might be related to anoxia or exogenous). These cases remained

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HISTORICAL BACKGROUND

THE SYNDROME of "encephalopathy and fatty degeneration of the viscera" was first described as a specific disease entity from Australia by Reye, Morgan, and Baral in 1963.¹ They reported 20 children ranging in age from five months to eight and one-half years who developed a viral illness that progressed to delirium, fever, convulsions, vomiting, disturbed respiratory patterns, and coma. Many of the patients had elevated serum transaminases and hypoglycemia with low cerebrospinal fluid (CSF) glucose. Seventeen of their patients died (81%) and at autopsy the brains showed evidence of cerebral edema and there was diffuse fatty accumulation in the liver and kidney.

Although Reye has been credited with the first complete description of this disorder, a review of the literature suggests that it is not new. An association between influenza and subsequent development of "toxic" encephalopathy was made by Putnam² in 1895. In 1929, Brain et al.³ described a series of patients with encephalitis-like illness who had cerebral edema and fatty accumulation in the liver. In 1961, Lyon et al.⁴ discussed "acute encephalopathies of obscure origin in infants and children." Virtually all the cases they presented were of children with antecedent viral illnesses who developed vomiting, stupor and coma, respiratory alterations (usually hyperpnea followed by respiratory arrest), and seizures. The mortality rate was high (88%). Serum transaminases were not reported, and pathologic changes were confined to the brain, where diffuse cerebral edema and anoxic changes were observed. These authors suggested that the disorder might be related to an unidentified toxin (endogenous or exogenous). These cases resemble the disorder now known as

Reye's syndrome quite closely, and at least some of them may in fact represent the same disease.

Two other reports of the same clinicopathologic entity, one from the United States⁵ and the other from Australia,⁶ were published in the same year as Reye's report, but the disorder commonly bears his name. Following these initial publications, numerous reports from several countries appeared, corroborating the similar clinical, laboratory, and pathologic abnormalities Reye had summarized.

By 1967, the literature contained 83 cases, and a controversy was raised regarding the specificity of Reye's syndrome as a disease entity. This is reflected in a 1969 editorial in *Lancet*,⁷ which suggested that the syndrome of encephalopathy with fatty infiltration of the viscera might not have a single cause but that it might "represent a common reaction to a variety of (infectious or toxic) stimuli." Although the literature on Reye's syndrome since that editorial was published has been prolific, the cause or causes of Reye's syndrome remain obscure, and the editor's concluding statement still may be valid.

EPIDEMIOLOGY

Reye's syndrome affects infants and children of all age groups, and recently adult cases have been reported. There appear to be two age peaks at four and 11 years, although it is common any time between the ages of five and 15 years. There is no sex predilection. The disease has been reported in many racial and ethnic groups throughout the world.

"Epidemics" of Reye's syndrome cases often correspond to outbreaks of influenza infection and offer an opportunity for more careful epidemiologic studies. In 1974, 349 cases were reported to the Center for Disease Control in Atlanta between January and June during an influenza B epidemic.⁸ Forty of 50 states reported cases of Reye's syndrome but the greatest concentration of cases (48%) was in the Midwest. Based on these data, Reye's syndrome was found to be much more common than previously believed, and in fact was ranked as the second most common cause of death in virus-related central nervous system diseases of children. In 1979-80, 517 cases were reported with an estimated incidence of 0.82 per 100,000 population under 17 years of age. The highest incidences of the disease occurred in Michigan, Ohio, Georgia, Colorado, Wyoming, Iowa, Nebraska, and South Dakota. However, reporting was voluntary and may be incomplete. A projected incidence based on these reports is 1.3 to 2.7 cases per 100,000 per year in children under the age of 17.

In the 1974 study, 94% of patients were white, 4% black, and 2% other minorities. Only 4.6% were under one year of age. Re-

cent reports^{9, 10} suggest that Reye's syndrome is more common than these figures indicate and may be misdiagnosed because the clinical picture is different in older children. An interesting epidemiologic feature has been observed in this respect. When the syndrome is observed in this respect. When the syndrome are typically white, middle class urban dwellers, infants under one year of age, and from other minority groups, inner-city and lower socioeconomic groups. This observation has led Trauner⁹ to draw a parallel between epidemic poliomyelitis prior to immunization and Reye's syndrome. This observation may be related to differences in sanitation with infants from lower socioeconomic groups at an earlier age to the virus. It is possible that Reye's syndrome results from initial exposure to the virus. This could explain the socioeconomic distribution of the disease.

An interesting and perhaps significant observation has been made about the onset of Reye's syndrome.¹¹ The interval between appearance of the encephalopathy of Reye's syndrome is typically three to five days. The reason for this is not clear, but closer studies of varicella infection and its prodromal period may provide insight into a possible mechanism.

Although most cases of Reye's syndrome follow a viral infection, some cases appear to follow bacterial infections.¹² As with varicella, there is a delay between vaccination and appearance of the disease. In one instance the interval is one to two weeks. Whether the live virus vaccines act as a trigger for the syndrome or merely interact with other factors in the body has yet to be determined.

Isolated cases of "recurrent" Reye's syndrome have been reported in the literature from time to time. However, other diseases that might produce similar symptoms have been completely ruled out. Disorders that have been ruled out include recurrent Reye's syndrome, inborn errors of metabolism, and organic acid metabolism, salicylate toxicity, and deficiency. Any child who has recurrent encephalopathy with liver dysfunction deserves a thorough evaluation, including salicylate levels, organic acids, and serum amino acids. The challenge between acute episodic encephalopathy and Reye's syndrome is suggested by isolated reports of recurrent episodes.

A genetic predisposition to Reye's syndrome is suggested by isolated reports of familial development of the disorder. However, the

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were under one year of age. Re-

cent reports^{9, 10} suggest that Reye's syndrome in infants is more common than these figures indicate and that infant cases might be misdiagnosed because the clinical presentation differs from that in older children. An interesting epidemiologic phenomenon has been observed in this respect. Whereas children with Reye's syndrome are typically white, middle class, and rural or suburban dwellers, infants under one year are more often black or from other minority groups, inner-city dwellers, and from lower socioeconomic groups. This observation led Huttenlocher and Trauner⁹ to draw a parallel between Reye's syndrome and epidemic poliomyelitis prior to immunization. The latter disease had a similar epidemiologic picture, which was thought to be related to differences in sanitation and to living conditions, with infants from lower socioeconomic situations being exposed at an earlier age to the virus. It is possible that Reye's syndrome results from initial exposure to a particular virus. This could explain the socioeconomic/ethnic differences observed.

An interesting and perhaps significant observation has been made about the onset of Reye's syndrome with varicella infection.¹¹ The interval between appearance of the rash and development of the encephalopathy of Reye's syndrome is almost uniformly three to five days. The reason for this specific interval is not clear, but closer studies of varicella patients during this time period may provide insight into a possible etiology for the disorder.

Although most cases of Reye's syndrome follow a natural viral infection, some cases appear to follow vaccination with live viruses.¹² As with varicella, there is a specific interval between vaccination and appearance of the encephalopathy, but in this instance the interval is one to two weeks following vaccination. Whether the live virus vaccines actually precipitate Reye's syndrome or merely interact with other natural infectious agents or toxins has yet to be determined.

Isolated cases of "recurrent" Reye's syndrome appear in the literature from time to time. However, in none of the reports have other diseases that might present a similar clinical picture been completely ruled out. Disorders that can masquerade as recurrent Reye's syndrome include inborn errors of urea cycle and organic acid metabolism, salicylism, and systemic carnitine deficiency. Any child who has recurrent episodes of encephalopathy with liver dysfunction deserves an extensive diagnostic evaluation, including salicylate levels, liver biopsy, urinary organic acids, and serum amino acid quantitations, and a protein challenge between acute episodes.

A genetic predisposition to the development of Reye's syndrome is suggested by isolated case reports of siblings who develop the disorder. However, these children may have contracted

Reye's syndrome as the result of common exposure to an environmental toxin, although none has been identified in familial cases thus far.

CLINICAL FEATURES OF REYE'S SYNDROME

CHILDREN OVER ONE YEAR OF AGE

The primary clinical features of this disorder are remarkably uniform in children from one to 16 years. The disease has two phases, a prodromal illness and an encephalopathic stage. A prodromal illness can be documented in virtually all well-studied cases. This prodrome consists of a viral infection, either upper respiratory infection or gastroenteritis. In approximately 20% of cases the prodromal disease is varicella. In "epidemic" Reye's syndrome, the most common infectious agent is influenza B. Numerous other viruses have been associated with sporadic cases (see below).

The prodrome may be quite mild. There is no association between the severity of the viral illness and subsequent development of encephalopathy. The child usually is recovering from this illness when he develops repetitive vomiting. There is no apparent gastrointestinal disturbance to explain the vomiting and most likely it is secondary to irritation of brain stem vomiting centers. The vomiting is so constant a feature of the child with Reye's syndrome that its absence makes the diagnosis questionable. Within 24-48 hours, the child goes from vomiting to behavioral changes, including delirium, combativeness and irrational behavior, disorientation, and hallucinations. Lethargy and stupor may alternate with periods of combativeness. Hyperventilation also may be prominent and likely is caused by primary stimulation of medullary respiratory centers. At this point, the child may begin to recover spontaneously or may deteriorate further into obtundation or coma. Seizures can occur at any time during the encephalopathic phase. Respiratory difficulties may develop and sudden respiratory arrest is not unusual.

The level of central nervous system dysfunction follows a rostral caudal progression from stupor to coma with intact brain stem function, then to decorticate or decerebrate posturing, and finally to a flaccid and areflexic state. Pupillary changes are prominent; in the early stages, pupils are dilated and sluggishly responsive to light; later, they may become asymmetric or fixed and dilated. Papilledema rarely is observed. Significant hyperthermia is quite frequent; there usually is no evidence of active infection and the fever probably is caused by hypothalamic dysfunction. Gastrointestinal bleeding often is present but not severe enough to cause significant blood loss.

Hepatomegaly is found in approximately 50% of cases. Laboratory evidence of liver dysfunction is present but jaundice is conspicuously absent. Hemorrhagic necrosis of the pancreas is present in some cases. At times, the pancreatic necrosis may produce hemorrhagic necrosis and

Sudden changes in the clinical picture may improve dramatically within a few days but may be followed by a rapid deterioration from delirium and brain stem dysfunction to respiratory collapse may occur over a few days. In children with Reye's syndrome, even constant observation in an intensive care unit may be necessary.

INFANTS UNDER ONE YEAR OF AGE

The clinical features of Reye's syndrome in infants under one year of age are somewhat different and thus make early recognition difficult, though present, usually is mild. The presenting problem in many infants is seizures, which may be the only sign of the encephalopathic stage. Apneic episodes may occur in the disease course, and other signs, especially hyperventilation, are common. Vomiting with Reye's syndrome may have a minimal vomiting but marked respiratory disturbances. All of these signs and symptoms of suspicion is necessary to consider the diagnosis.

The great majority of infants with Reye's syndrome have hepatomegaly, and all have evidence of liver dysfunction on laboratory tests. In contrast to older children, infants under one year of age have a difficult time reversing the disease. This is one of the reasons the incidence of seizures in the infant is high. The cerebrospinal fluid is normal in most cases, and the cerebrospinal fluid is normal in the cerebrospinal fluid and, at times, elevated.

TABLE 1.—FEATURES OF REYE'S SYNDROME

Epidemiology—poor, city dwellers
Clinical presentations—vomiting, lethargy, seizures
Respiratory disturbances—hyperventilation
Seizures frequent and early

common exposure to an environment has been identified in familial

3 SYNDROME

E

f this disorder are remarkably 16 years. The disease has two an encephalopathic stage. A ated in virtually all well-stud- of a viral infection, either up- troenteritis. In approximately ase is varicella. In "epidemic" on infectious agent is influenza been associated with sporadic

ld. There is no association be- ness and subsequent develop- ild usually is recovering from petitive vomiting. There is no ance to explain the vomiting o irritation of brain stem vom- constant a feature of the child absence makes the diagnosis s, the child goes from vomiting ; delirium, combativeness and 1, and hallucinations. Lethargy riods of combativeness. Hyper- nt and likely is caused by pri- ratory centers. At this ove. spontaneously or may de- or coma. Seizures can occur at hic phase. Respiratory difficul- ratory arrest is not unusual. stem dysfunction follows a ros- por to coma with intact brain e or decerebrate posturing, and c state. Pupillary changes are upils are dilated and sluggishly ay become asymmetric or fixed is observed. Significant hyper- usually is no evidence of active is caused by hypothalamic dys- ng often is present but not se- blood loss.

Hepatomegaly is found in approximately 85% of patients; laboratory evidence of liver dysfunction virtually always is present, but jaundice is conspicuously absent. The incidence of pancreatitis in Reye's syndrome ranges from 0% to 22% of autopsied cases. At times, the pancreatic involvement is so severe as to produce hemorrhagic necrosis and death.

Sudden changes in the clinical status are common. Patients may improve dramatically within a few hours or they may plateau for many days before improvement is observed. Likewise, rapid deterioration from delirium through the stages of coma and brain stem dysfunction to respiratory arrest and cardiovascular collapse may occur over a few hours. For this reason, all children with Reye's syndrome, even in the early stages, require constant observation in an intensive care unit setting until awake.

INFANTS UNDER ONE YEAR OF AGE (Table 1)

The clinical features of Reye's syndrome in infants under one year of age are somewhat different from those of older children and thus make early recognition more difficult. Vomiting, although present, usually is mild. Diarrhea is frequent. The presenting problem in many infants with Reye's syndrome is seizures, which may be the initial manifestation of the encephalopathic stage. Apneic episodes are common even early in the disease course, and other respiratory abnormalities, especially hyperventilation, are quite prominent. Thus, infants with Reye's syndrome may have a prodromal illness followed by minimal vomiting but marked seizure activity and respiratory disturbances. All of these signs are nonspecific, and a high index of suspicion is necessary to consider the diagnosis of Reye's syndrome.

The great majority of infants with Reye's syndrome have hepatomegaly, and all have evidence of liver dysfunction on laboratory tests. In contrast to older children, virtually all of the infants under one year of age have hypoglycemia that may be difficult to reverse. This is one probable reason for the increased incidence of seizures in the infant age group. Examination of the cerebrospinal fluid is normal with the exception of hypoglycorrhachia and, at times, elevated pressure.

TABLE 1.—FEATURES OF INFANTS WITH REYE'S SYNDROME

Epidemiology—poor, city dwellers, ethnic minorities
Clinical presentations—vomiting may be minimal or absent
Respiratory disturbances (apnea or hyperventilation) prominent
Seizures frequent and early in course

ADULTS WITH REYE'S SYNDROME

Isolated cases of adult-onset Reye's syndrome have been reported only recently. Except for one case report of a 51-year-old woman, most of the patients have been between 20 and 30 years of age. The clinical picture is similar to that seen in children, with a viral illness followed by persistent vomiting, delirium, lethargy, and coma. Hyperammonemia and evidence of liver dysfunction with elevated transaminases are present but have been relatively mild in the few cases reported. Serum bilirubin concentrations tend to be mildly elevated (up to 7.5 mg/dl). Liver biopsy confirmation of microvesicular fatty accumulation has been documented in some cases. It appears that Reye's syndrome is not limited to the pediatric population and must be considered in patients of any age who develop vomiting and encephalopathy following a viral illness.

PATHOLOGY

Pathologic changes have been described in numerous organs, including liver, brain, kidney, heart, and pancreas. Grossly, the liver is enlarged and often yellowish. The most prominent light microscopic change is diffuse microvesicular accumulation of lipid within the cytoplasm of hepatocytes (Fig 1). These droplets do not usually displace the nucleus from its central location within the cell. Fat stains such as oil red O and Sudan black performed on frozen sections of tissue best demonstrate this abnormality. Liver cell cytoplasm appears foamy when examined after hematoxylin and eosin stains. Glycogen stains such as periodic acid-Schiff (PAS) reveal diffuse depletion of glycogen. Hepatocellular necrosis and inflammatory infiltrates are notably absent in this disorder.

Ultrastructural abnormalities are most marked in the mitochondria,¹³ which are swollen and pleomorphic with a flocculent matrix; the outer membrane is deformed by cytoplasmic invaginations and the cristae may be fragmented. Intramitochondrial dense bodies are absent. Other changes in intracellular structures include proliferation of the smooth endoplasmic reticulum, depletion of glycogen, and an increased number of peroxisomes. In addition, lipid droplets are present in the cytoplasm.

The brain is grossly swollen, with flattening of gyri, and the brain weight is increased relative to normal for age. Histologically, cerebral edema is apparent. Changes consistent with anoxia, namely, focal neuronal loss, eosinophilic changes in neurons, and glial proliferation, sometimes are present. Ultrastructural changes include accumulation of edema fluid in glial cells and focal areas of swelling in myelin sheaths (myelin "bleb" formation). Variable changes have been found within

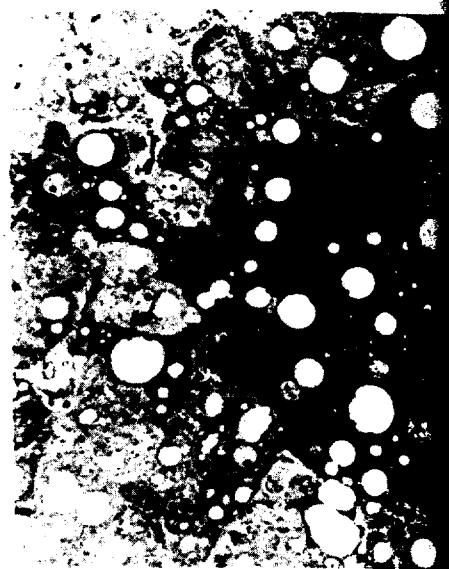


Fig 1.—Thick section of liver from patient with diffuse lipid accumulation within hepatocytes stained with toluidine blue; $\times 500$.

brain mitochondria, consisting of swelling and swelling.

Heart, kidney, and skeletal muscle lipid accumulation. Mitochondria found in the liver have been observed in skeletal muscle as well. The pancreas shows necrosis, hemorrhage, and inflammatory infiltrations of an unknown type in the pancreatic tissue in the absence of inflammation.

BIOCHEMICAL ABNORMALITIES

In Reye's original description of the presence of elevated serum transaminases, characteristic laboratory abnormalities, other metabolic derangements have been reported. Huttenlocher et al.¹⁴ first reported hypoglycemia in Reye's syndrome and serum ammonia elevation might be the clinical disease. This has been investigated, and it appears that serum ammonia greater than 350 $\mu\text{g/dl}$ are associated with poor terms of survival.

Hyperammonemia is present in Reye's syndrome early in the course of the disease.

t Reye's syndrome have been reported. One case report of a 51-year-old woman between 20 and 30 years similar to that seen in children, by persistent vomiting, delirium, anememia and evidence of liver transaminases are present but have few cases reported. Serum bilirubin is elevated (up to 7.5 mg/dl). Liver shows vesicular fatty accumulation. It appears that Reye's syndrome is a population and must be considered as a complication of vomiting and encephalopathy.

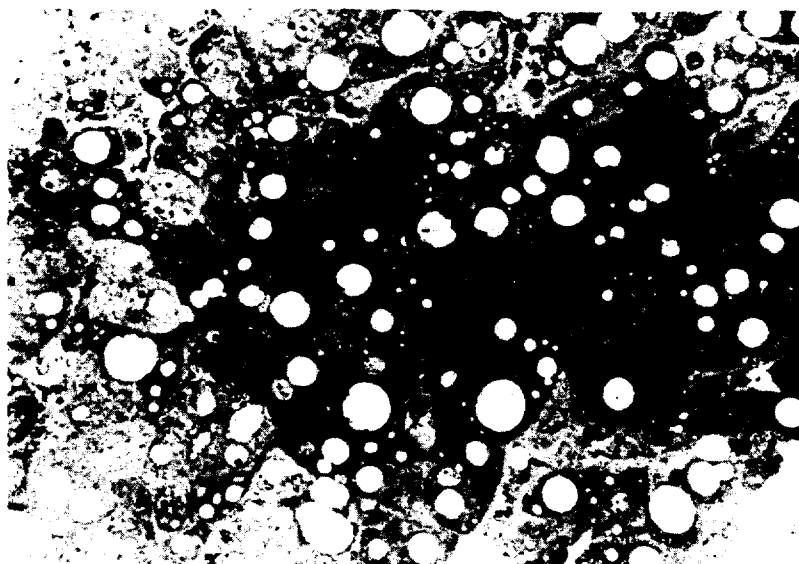


Fig 1.—Thick section of liver from patient with Reye's syndrome demonstrating diffuse lipid accumulation within hepatocytes. Tissue was embedded in Epon and stained with toluidine blue; $\times 500$.

as described in numerous organs, heart, and pancreas. Grossly, the liver is pale and edematous. The most prominent light microvesicular accumulation of lipid within hepatocytes (Fig 1). These droplets displace the nucleus from its central location and are best demonstrated by oil red O and Sudan black stains. The liver tissue best demonstrate this abnormality. The liver appears foamy when examined grossly. Glycogen stains such as periodic acid-Schiff demonstrate depletion of glycogen. Hemorrhagic infiltrates are notably

most marked in the mitochondria. Mitochondria are amorphous with a flocculent appearance, deformed by cytoplasmic invagination and fragmented. Intramitochondrial changes in intracellular structure include smooth endoplasmic reticulum, increased number of peroxisomes, and vesicles present in the cytoplasm.

There is flattening of gyri, and the liver returns to normal for age. Histologically, changes consistent with cell loss, eosinophilic changes in the cytoplasm, and sometimes are present. Accumulation of edema fluid in the interstitium and myelin sheaths (myelin vacuolization) have been found within

brain mitochondria, consisting primarily of matrix distortion and swelling.

Heart, kidney, and skeletal muscle all contain evidence of lipid accumulation. Mitochondrial changes similar to those found in the liver have been observed in myocardium and skeletal muscle as well. The pancreas may show evidence of focal necrosis, hemorrhage, and inflammatory changes. Intranuclear inclusions of an unknown type have been demonstrated in pancreatic tissue in the absence of inflammatory changes.

BIOCHEMICAL ABNORMALITIES

In Reye's original description of the disease he emphasized the presence of elevated serum transaminases and hypoglycemia as characteristic laboratory abnormalities. Since then, numerous other metabolic derangements have been documented (Fig 2). Huttenlocher et al.¹⁴ first reported the presence of hyperammonemia in Reye's syndrome and suggested that the degree of serum ammonia elevation might correlate with the severity of the clinical disease. This has been corroborated by other investigators, and it appears that serum ammonia concentrations greater than 350 $\mu\text{g/dl}$ are associated with a poor prognosis in terms of survival.

Hyperammonemia is present in virtually all patients with Reye's syndrome early in the course of the illness. This abnormality

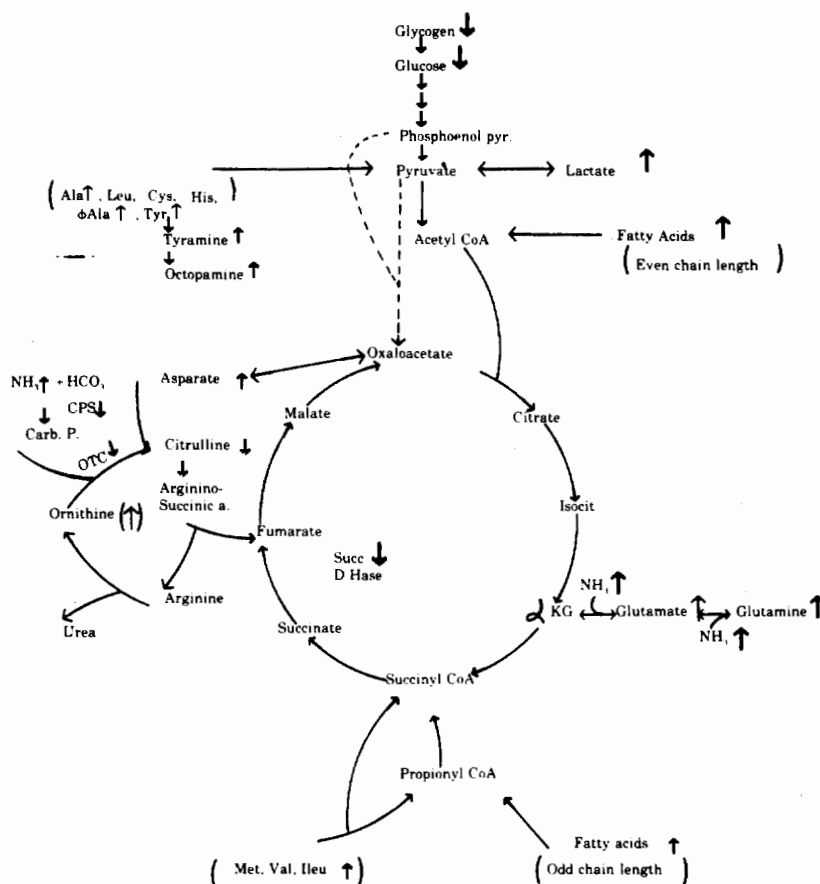


Figure 2.—Diagram of metabolic derangements in Reye's Syndrome.

malinity is transient, however, and ammonia levels usually return to normal within 24–48 hours, regardless of the treatment involved. Transient reductions in enzyme activity of the mitochondrial components of the urea cycle, ornithine transcarbamoylase and carbamyl phosphate synthetase, have been documented and may explain the hyperammonemia. In addition, a catabolic state exists secondary to the viral illness, anorexia, and vomiting, resulting in an increased release of amino acids from muscle, which may also contribute to the hyperammonemia.

Serum transaminases always are abnormally elevated whereas bilirubin concentrations are characteristically normal or minimally elevated. In fact, if total bilirubin concentration is greater than 5 mg/dl, other diagnostic possibilities should be considered.

Hypoglycemia was stressed in early reports, but studies of large series of patients have revealed that hypoglycemia occurs in only about 40% of patients and primarily in children under

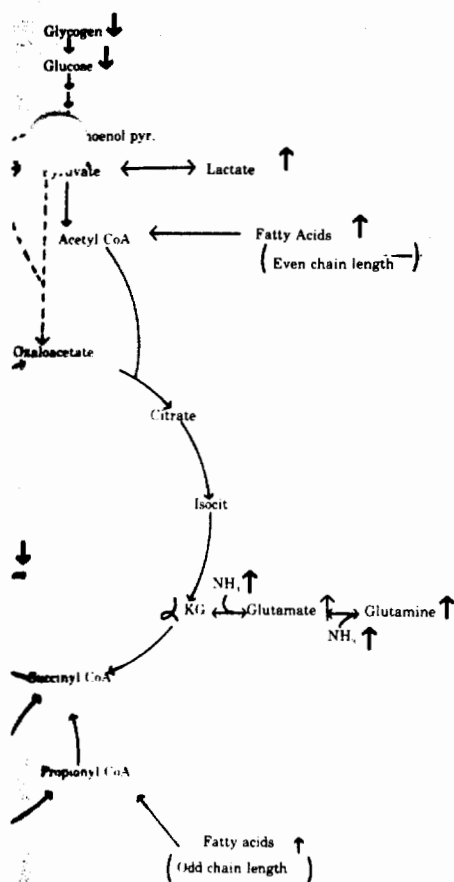
four years of age.¹⁵ For this useful or necessary criterion mechanism of the hypoglycemia related to a defect in hepaticities of pyruvate dehydrogenase has been found in patients with tro studies of liver obtained have documented a defect in gluconeogenesis. Both of these findings support a defect in gluconeogenesis.

Lactic acidemia is a frequent finding in patients with Reye's syndrome, suggesting impaired oxidative metabolism by extrahepatic tissues.¹⁶ have demonstrated decreased levels of lactate in the brains of patients with Reye's syndrome, at least in part, to mitochondrial dysfunction.

Elevations of total serum fatty acids are found in some patients with Reye's syndrome. In addition, specific elevations (propionate, butyrate, isobutyrate) have been documented. Furthermore, clearance of these substances is decreased, which may relate with clinical improvement. Improvements include an increase in appetite, resolution of vomiting, or a block in mitochondrial fatty acid oxidation.

Brain concentrations of norepinephrine, dopamine, and glutamate are elevated in patients with Reye's syndrome. Abnormalities have also been reported in hepatic encephalopathy regarding neurotransmission. In association with the elevated concentrations of norepinephrine and tyramine concentration, the onset of coma. Serum prolactin has been suggested that the hypothalamic dopamine precursor of octopamine, tyramine, concentrations may reflect the increased ventricular fluid found in Reye's syndrome, suggesting a release of dopamine into the cerebrospinal fluid.

A significant increase in levels of lysine, glutamine, and glutamate has been found in patients. This pattern is specific for Reye's syndrome.



Metabolic derangements in Reye's Syndrome.

Ammonia levels usually return to normal, regardless of the treatment initiated. In enzyme activity of the mitochondrial cycle, ornithine transcarbamoylase deficiency, have been documented and hyperammonemia. In addition, a catabolic state of illness, anorexia, and vomiting, release of amino acids from muscle, and the hyperammonemia. Amino acids are abnormally elevated whereas bilirubin concentration is greater than normal. In early reports, but studies of hypoglycemia occurs and primarily in children under

four years of age.¹⁵ For this reason, it no longer is considered a useful or necessary criterion for diagnosis of the disease. The mechanism of the hypoglycemia is not clear but is thought to be related to a defect in hepatic gluconeogenesis. Decreased activities of pyruvate dehydrogenase and pyruvate carboxylase have been found in patients with Reye's syndrome. In addition, in vitro studies of liver obtained from children with Reye's syndrome have documented a defect in glucose formation from pyruvate. Both of these findings support the hypothesis that there may be a defect in gluconeogenesis.

Lactic acidemia is a frequent abnormality that may be related to impaired oxidative metabolism of glucose or to accelerated production by extrahepatic tissues such as muscle. Shannon et al.¹⁶ have demonstrated de novo production of lactate in the brains of patients with Reye's syndrome. This may be related, at least in part, to mitochondrial injury.

Elevations of total serum free fatty acids and nonesterified fatty acids are found in some patients with Reye's syndrome. In addition, specific elevations of serum short-chain fatty acids (propionate, butyrate, isobutyrate, valerate, isovalerate, octanoate) have been documented during the acute illness. Furthermore, clearance of these short-chain fatty acids appears to correlate with clinical improvement.¹⁷ Possible causes for the fatty acidemia include an increased release from adipose tissue as a result of anorexia, vomiting, or a lipolytic response to a virus—or a block in mitochondrial beta oxidation.

Brain concentrations of a putative neurotransmitter, octopamine, are elevated in patients with Reye's syndrome and concentrations of norepinephrine and dopamine are diminished. These abnormalities have also been documented in patients with hepatic encephalopathy regardless of the etiology. It has been suggested that one mechanism for the encephalopathy may be that octopamine acts as a false neurotransmitter and blocks normal neurotransmission. In association with this, plasma tyramine concentrations are elevated in patients with Reye's syndrome, and tyramine concentrations appear to correlate with the duration of coma. Serum prolactin levels also are elevated. It has been suggested that the hyperprolactinemia might be related to hypothalamic dopamine depletion.¹⁸ Also, since tyramine is a precursor of octopamine, the elevation of serum tyramine concentrations may reflect the elevation in brain octopamine levels. Increased ventricular fluid concentrations of dopamine are also found in Reye's syndrome, suggesting an increased neuronal release of dopamine into the ventricular fluid.

A significant increase in serum concentrations of the amino acids lysine, glutamine, alanine, and α-amino-n-butyrate is found in patients. This pattern of aminoacidemia appears to be specific for Reye's syndrome and may serve as a diagnostic tool.¹⁹

Potential causes for this abnormality include increased release from muscle secondary to a catabolic state and inhibition of hepatic uptake and/or metabolism of amino acids. CSF glutamine concentrations also are elevated and may reflect the brain's attempt to clear excess ammonia.

Creatine phosphokinase concentrations are elevated and isoenzyme determinations reveal skeletal muscle or cardiac components or both to be involved. It has been suggested in studies of a small number of patients that those patients with elevations of both skeletal muscle and cardiac isoenzymes have a poorer prognosis than those with abnormalities in either one of these enzymes alone.

Elevations in serum uric acid concentrations have been observed many times, but are of interest now because of Aprille's observation that a serum factor²⁰ from patients with Reye's syndrome that inhibits liver mitochondrial respiration appears to be uric acid. The cause of the uric acid elevation and the metabolic consequences of this defect are not clear.

Hyperosmolality is a frequent complication in Reye's syndrome patients and usually is caused by a combination of dehydration and the use of osmotic diuretics plus hypertonic glucose. Hyperthermia is present in virtually all patients early in the disease course and is unrelated to an obvious infectious site. The temperature abnormality most likely is the result of hypothalamic dysfunction. Likewise, respiratory alkalosis is present on a central basis as a result of primary stimulation of respiratory centers in the brain stem. Often a mixed metabolic acidosis is observed in addition to the respiratory alkalosis. Hypotension may occur early as a result of dehydration or late in the disease as a complication of brain stem compression.

Renal abnormalities usually are not severe and typically consist of elevations in blood urea nitrogen concentrations. This may be a response to dehydration or to a hyperosmolar state, or may reflect direct involvement of the kidneys with fatty infiltration. Occasional cases of transient acute renal failure in the face of positive fluid balance and adequate central venous pressure have been reported recently. The cause of the renal failure was not clear, but in most cases reversed and had no apparent effect on outcome.

Elevated serum amylase concentrations suggestive of pancreatitis are found occasionally. Liver glycogen stores are depleted and carbohydrate metabolism is impaired. Serum insulin concentrations may be either elevated or depressed in the acute stages of Reye's syndrome. Survivors of this disease appear to have impaired responsiveness to endogenous insulin during oral glucose tolerance tests.²¹ This observation suggests that individuals with Reye's syndrome may have an alteration in endogenous insulin such that it is less effective than normal. This pos-

sibility has implications for the acute illness.

Prothrombin time is prolonged. Platelet count usually is normal and decreased coagulability also have been described. Thrombotic lesions sent from the circulation are not reported. Intravascular coagulation is not recorded, this certainly is not a function of vitamin K to pat- ineffective, and fresh-frozen plasma and completed coagulation factors.

Serum complement abnormalities are present in a small number of patients. Consistent early reduction of complement prior to treatment suggests the possibility of immune complex disease or the complement system. The presence of immune complex

DIAGNOSIS OF REYE'S

The diagnosis of Reye's syndrome is based on clinical and laboratory data. This diagnosis is made in the presence of an antecedent viral infection, progression to lethargy, hyperventilation, delirium, obtundation, or coma, and laboratory evidence of dysfunction with hyperammonemia, hypoglycemia, and normal bilirubin; and absence of liver disease might mimic Reye's syndrome.

A controversy has existed as to the necessity for documenting liver disease in order to diagnose Reye's syndrome. The course and laboratory data over one year of age that are consistent with Reye's cases. Indications for liv-

TABLE 2.

1. Clinical progression of disease with behavioral changes.
2. Laboratory evidence of hypoglycemia, hyperammonemia, and normal bilirubin of hepatogenic cause.
3. Exclusion of CNS infection.
4. Liver biopsy in doubtful cases.

normality include increased release of amino acids. CSF glutamine may reflect the brain's at-

concentrations are elevated and iso-

skeletal muscle or cardiac com-
It has been suggested in studies
that those patients with elevations
of cardiac isoenzymes have a poorer
normalities in either one of these

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from patients with Reye's syn-
dromal respiration appears to
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are not clear.

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drome caused by a combination of dehy-
dration plus hypertonic glucose.
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disease have an obvious infectious site. The
likely is the result of hypota-
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and a mixed metabolic acidosis is
piratory alkalosis. Hypotension
dehydration or late in the disease
compression.

are not severe and typically con-
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tion acute renal failure in the face
of adequate central venous pressure
is a cause of the renal failure was
ruled out and had no apparent effect

concentrations suggestive of pan-
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pleted. Serum insulin
is elevated or depressed in the acute
stages of this disease appear to
be endogenous insulin during oral
administration suggests that individ-
uals have an alteration in endoge-
nous insulin than normal. This pos-

sibility has implications for the treatment of fatty acidemia in
the acute illness.

Prothrombin time is prolonged in all patients with Reye's syn-
drome. Platelet count usually is normal. Hypofibrinogenemia
and decreased coagulation factor levels except for factor VIII
also have been described. Fibrin split products usually are ab-
sent from the circulation. Although rare accounts of dissemi-
nated intravascular coagulation in Reye's syndrome have been
recorded, this certainly is not typical of the disease. Administra-
tion of vitamin K to patients with Reye's syndrome usually is
ineffective, and fresh-frozen plasma is required to restore de-
pleted coagulation factors.

Serum complement abnormalities also have been observed in
a small number of patients with Reye's syndrome. There is a
consistent early reduction in serum C1 activity and C1 S concen-
trations prior to treatment. These abnormalities may be the re-
sult of interruption in synthesis of these proteins or may suggest
the possibility of immune complex depletion of components of
the complement system. However, to date there is no other evi-
dence of immune complex formation in this disease.

DIAGNOSIS OF REYE'S SYNDROME (TABLE 2)

The diagnosis of Reye's syndrome rests primarily on historical
and clinical data. This disorder should be suggested by a history
of an antecedent viral illness followed by vomiting, with pro-
gression to lethargy, hyperexcitability, and agitation, then to
delirium, obtundation, or coma; laboratory evidence of hepatic
dysfunction with hyperammonemia, abnormal liver function
tests and normal bilirubin, and a normal cerebrospinal fluid ex-
amination; and absence of other diseases (e.g., salicylism) that
might mimic Reye's syndrome.

A controversy has existed for several years regarding the ne-
cessity for documentation of typical pathologic changes in the
liver in order to diagnose Reye's syndrome. However, the clinical
course and laboratory abnormalities are so typical in children
over one year of age that a liver biopsy is not necessary in most
cases. Indications for liver biopsy recently were outlined at the

TABLE 2.—DIAGNOSIS OF REYE'S SYNDROME

1. Clinical progression of prodromal viral illness to vomiting, with
behavioral changes, delirium, or coma
2. Laboratory evidence of hepatic dysfunction, with
hyperammonemia, elevation of serum transaminase, depression
of hepatogenic coagulation factors, and normal bilirubin
3. Exclusion of CNS infection and toxin ingestion by appropriate tests
4. Liver biopsy in doubtful or atypical cases

National Institutes of Health Consensus Development Conference on the Diagnosis and Treatment of Reye's Syndrome (Table 3). A liver biopsy should be performed in the following situations: (1) children under one year of age, (2) atypical cases (e.g., without antecedent viral infection or vomiting), (3) when new and potentially dangerous therapeutic regimens are being considered. A percutaneous liver biopsy has been found to be a safe procedure with minimal morbidity in patients with Reye's syndrome.

The differential diagnosis of Reye's syndrome is not extensive (Table 4). However, several diseases may mimic Reye's syndrome, at least initially. Central nervous system infections, either viral or bacterial, may follow an upper respiratory infection and produce vomiting and lethargy or obtundation. This diagnosis can be ruled out rapidly by examination of the cerebrospinal fluid. Ingestion of various toxins may produce similar symptomatology. The most common agent is salicylate, which causes vomiting, seizures, obtundation, hyperventilation, hypoglycemia, and abnormal liver function tests. A serum salicylate level of 25 mg/dl or greater suggests salicylism rather than Reye's syndrome. The liver biopsy in salicylism may show fatty accumulation, but this is not microvesicular and usually not as prominent as the fatty changes in Reye's syndrome. Other toxins include aflatoxin, isopropyl alcohol, and valproic acid. In addition, several inborn metabolic defects may simulate Reye's syndrome. These include systemic carnitine deficiency, the urea cycle defects, and organic acid disorders such as glutaric aciduria and isovaleric acidemia. The last group of diseases should be considered especially in cases of recurrent Reye's-like illnesses and in familial cases.

TABLE 3.—INDICATIONS FOR LIVER BIOPSY IN SUSPECTED REYE'S SYNDROME

1. Infants under one year of age
2. Children with recurrent episodes
3. Familial cases
4. Sporadic cases without infection or vomiting
5. If new and potentially dangerous therapeutic regimens are planned

TABLE 4.—DIFFERENTIAL DIAGNOSIS OF REYE'S SYNDROME

1. Meningitis
2. Varicella hepatitis/encephalitis
3. Toxins: salicylates, methyl bromide, hypoglycin, isopropyl alcohol, aflatoxin, lead, valproic acid
4. Anoxic encephalopathy
5. Inborn metabolic defects: systemic carnitine deficiency, hyperammonemia syndromes, organic acid disorders

STAGING OF REYE'S SYNDROME

Since the treatment of severe Reye's syndrome is aggressive and involves potential risks, some means of staging the severity of the disease have been suggested, but the most widely used is that of Lovejoy et al.²² This system

- I. Vomiting and lethargy.
- II. Disorientation, delirium and response to noxious stimuli.
- III. Obtundation, coma, decorticate or decerebrate posturing, pupillary reflexes.
- IV. Coma, decerebrate rigidity, large fixed pupils.
- V. Absent deep tendon reflexes, acidosis.

In general, stages I and II are mild, stages III–V are "severe" cases requiring intensive care. In an attempt to implement a uniform staging system, all centers treating patients with Reye's syndrome have adopted the Statement formulated at the National Institutes of Health Consensus Development Conference on the Diagnosis and Treatment of Reye's Syndrome outlined in Table 5. This is shown in Table 5.

ETIOLOGY AND PATHOGENESIS

ASSOCIATION WITH VIRUSES

Numerous viruses have been implicated in the development of Reye's syndrome. The evidence of active viral infection in the dying of Reye's syndrome has been controversial. Children with Reye's syndrome have been exposed to a variety of viral infections, but the most commonly associated agents associated with the disease are influenza B and varicella. In some cases follow influenza B or varicella. In other cases involving other viruses are slightly more plausible. Another theory is that Reye's syndrome represents a reaction to a viral infection. This could explain the geographic distributions of infantile Reye's syndrome.

Several investigators have suggested that a viral infection leads to the development of Reye's syndrome. Animal studies have documented a similar syndrome in animals exposed to viruses plus certain insecticides. Viral potentiation of the effect of a particular 4-pentenol acid.

Development Confer-
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ni , (3) when new
gimens are being con-
een found to be a safe
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system infections, ei-
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may show fatty accu-
and usually not as
ndrome. Other toxins
alproic acid. In addi-
simulate Reye's syn-
ficiency, the urea cy-
as glutaric aciduria
of diseases should be
Reye's-like illnesses

STAGING OF REYE'S SYNDROME

Since the treatment of severe Reye's syndrome generally is aggressive and involves potential risks, it is important to use some means of staging the severity of the illness. Several staging systems have been suggested, but the most commonly used is that of Lovejoy et al.²² This system utilizes five stages:

- I. Vomiting and lethargy.
- II. Disorientation, delirium and combativeness, appropriate response to noxious stimuli.
- III. Obtundation, coma, decorticate posturing, preserved pupillary reflexes.
- IV. Coma, decerebrate rigidity, loss of oculocephalic reflexes, large fixed pupils.
- V. Absent deep tendon reflexes, respiratory arrest, and flaccidity.

In general, stages I and II are considered "mild" whereas stages III-V are "severe" cases requiring aggressive treatment. In an attempt to implement a uniform staging system for use by all centers treating patients with Reye's syndrome, the Consensus Statement formulated at the National Institutes of Health Consensus Development Conference on the Diagnosis and Treatment of Reye's Syndrome outlined a revised staging system. This is shown in Table 5.

ETIOLOGY AND PATHOGENESIS

ASSOCIATION WITH VIRUSES

Numerous viruses have been linked with the subsequent development of Reye's syndrome (Table 6). However, thus far, evidence of active viral infection in the liver or the brain of patients dying of Reye's syndrome has been lacking. It is possible that children with Reye's syndrome have a similar response to a variety of viral infections, but the large number of different viral agents associated with the disease makes this less likely. Most cases follow influenza B or varicella infections, and if sporadic cases involving other viruses are ignored, this hypothesis has slightly more plausibility. Another possibility is that Reye's syndrome represents a reaction to initial exposure to a particular virus. This could explain the differences in socioeconomic and geographic distributions of infant and childhood cases.

Several investigators have suggested that a virus-toxin interaction leads to the development of Reye's syndrome. Experimental studies have documented a Reye's-like illness in animals exposed to viruses plus environmental toxins, especially insecticides. Viral potentiation of certain chemical toxins, in particular 4-pentenoic acid, results in an experimental syn-

BIOSY IN

vomiting

d

YE'S SYNDROME

glycin, isopropyl

ne deficiency,
acid disorders

TABLE 5.—STAGING OF REYE'S SYNDROME

	I	II	III	IV	V
Level of consciousness	Lethargy; follows verbal commands	Combative/stupor; verbalizes inappropriately	Coma	Coma	Coma
Posture	Normal	Normal	Decorticate	Decerebrate	Flaccid
Response to pain	Purposeful	Purposeful/Sluggish	Decorticate	Decerebrate	None
Pupillary reaction	Brisk	Sluggish	Sluggish	Sluggish	None
Oculocephalic reflex (doll's eyes)	Normal	Conjugate deviation	Conjugate deviation	Inconsistent or absent	None

TABLE 6.—VIRUSES ASSOCIATED WITH REYE'S SYNDROME

Influenza B (most common)
Reye's
Varicella (20% of cases)
Influenza A ₁ and A ₂
Parainfluenza
Epstein-Barr virus
Echovirus
Coxsackie A, A ₁ , A ₂
Reovirus
Adenovirus
Rubella
Rubeola
Polio virus Type 1
Herpes simplex

drome of hypoglycemia, encephalopathy of the viscera quite similar to the disorder clinically identical to the disorder (alopathy) is produced by ingestion of a heat-stable metabolite of the toxin is found in grains and not in butter. Recently, several cases of Reye's syndrome in the United States have been associated with aflatoxin B₁. It is possible that the drome is related to an initial exposure to a toxin such as aflatoxin B₁ closely by a second hepatic infection with a virus agent such as influenza.

Another disorder quite similar to Reye's syndrome is acute hemorrhagic vomiting sickness, also is caused by a toxin, cin A, found in unripe ackee which develop vomiting, coma, seizures, and death. That both Jamaican vomiting sickness and Reye's syndrome have an identifiable etiology has stimulated a search for a common agent in these patients. Other than the case found in the liver, the only possible association with Reye's syndrome in the United States is with aspirin. This syndrome was much more common in children taking aspirin than in children not taking aspirin. These results suggest that aspirin is such a common cause of this syndrome is so rare. However, the withholding of this finding is common to withhold the use of salicylates.

Posture	Normal	Normal	Decorticate	Decerebrate	Flaccid
Response to pain	Pupillary	Pupillary	Decorticate	Decerebrate	None
Pupillary reaction	Normal	Normal	Sluggish	Sluggish	None
Oculocephalic reflex (doll's eyes)	Normal	Normal	Conjugate deviation	Inconsistent or absent	None

TABLE 6.—VIRUSES ASSOCIATED WITH REYE'S SYNDROME

Influenza B (most common in "epidemic" Reye's)
Varicella (20% of cases)
Influenza A ₁ and A ₂
Parainfluenza
Epstein-Barr virus
Echovirus
Coxsackie A, A ₁ , B ₁ , and B ₄
Reovirus
Adenovirus
Rubella
Rubeola
Polio virus Type I
Herpes simplex

drome of hypoglycemia, encephalopathy, and fatty degeneration of the viscera quite similar to Reye's syndrome.²³ In Thailand, a disorder clinically identical to Reye's syndrome (Udorn encephalopathy) is produced by ingestion of aflatoxin B₁.²⁴ This is a heat-stable metabolite of the fungus *Aspergillus flavus*. Aflatoxin is found in grains and nuts and, in particular, stale peanut butter. Recently, several cases of Reye's syndrome in the United States have been associated with high liver concentrations of aflatoxin B₁. It is possible that the pathogenesis of Reye's syndrome is related to an initial insult to the liver; for example, an exposure to a toxin such as aflatoxin or insecticide, followed closely by a second hepatic insult; for example, from an infectious agent such as influenza B.

Another disorder quite similar to Reye's syndrome, Jamaican vomiting sickness, also is caused by an ingested toxin, hypoglycin A, found in unripe ackee fruit. Children who eat this fruit develop vomiting, coma, seizures, and hypoglycemia. The fact that both Jamaican vomiting sickness and Udorn encephalopathy have an identifiable exogenous toxin as an etiologic agent has stimulated a search for similar toxins in Reye's syndrome patients. Other than the case reports in which aflatoxin was found in the liver, the only other toxic agent found to have a possible association with Reye's syndrome in epidemiologic studies in the United States is salicylates.²⁵ In recent studies, Reye's syndrome was much more likely to develop in children given aspirin than in children treated with acetaminophen for the same infection. These results have been disputed on the basis that aspirin is such a commonly used medication and Reye's syndrome is so rare. However, until a more thorough examination of this finding is completed, it would appear prudent to withhold the use of salicylates to control fever and other symp-

toms of viral illness in children, in particular those symptoms associated with influenza or varicella infection.

Using this hypothesis as a basis, an attempt was made to reproduce Reye's syndrome in ferrets by simultaneous exposure to virus and salicylates.²⁶ This experiment failed to demonstrate the typical pathologic lesions of Reye's syndrome. Animals were infected by the nasal route with influenza A/Hong Kong or influenza B virus and given gastric feedings of large doses of salicylates. Although these maneuvers produced some depression of ornithine transcarbamoylase activity and mild fatty changes in the liver, salicylates did not potentiate the effects of the virus nor were the pathologic changes similar enough to those found in Reye's syndrome to constitute an experimental model for the disease. This study does not necessarily rule out a role for salicylates in the development of the human disease, however.

In a search for potential toxins, a low molecular weight, heat-stable factor has been isolated from serum of patients with Reye's syndrome. This serum factor inhibits mitochondrial respiratory activity in vitro. There is some controversy about the identity of this factor. Aprille's work with rat liver mitochondria suggested that uric acid is the toxic agent whereas Ansevin²⁷ believes that short-chain fatty acids are the serum factors, at least in studies using rat brain mitochondria. Although serum uric acid concentrations are elevated in patients with Reye's syndrome, it is unlikely that this substance plays a significant role in the pathogenesis of the disease. On the other hand, short-chain fatty acids, also elevated in Reye's syndrome, have been shown to produce mitochondrial swelling in vitro and to inhibit mitochondrial respiratory activity. These agents may well be the "serum factor" described.

ROLE OF AMMONIA

Hyperammonemia is present in virtually all patients with Reye's syndrome early in the course of the illness. Several investigators have found a correlation between the degree of hyperammonemia and the outcome of the disease, although this has not been found uniformly. Elevations of serum ammonia concentrations in experimental animals are capable of producing coma. Thus, ammonia is an endogenous toxin that might be responsible for the encephalopathy in Reye's syndrome.

There are arguments against the hypothesis that ammonia is the primary or sole toxin. The first is that serum ammonia concentrations often return to normal within 24-48 hours of the onset of encephalopathy whereas clinical symptoms may continue to worsen. The second is that ammonia infusion into experimental animals requires extremely high serum concentrations (on the order of 2,000 $\mu\text{g}/\text{dl}$) before coma is induced.²⁸ In

normal human subjects, blood ammonia raised to greater than 700 $\mu\text{g}/\text{dl}$ with changes. Although concentrations of ammonia with Reye's syndrome vary widely, they are of 150-500 $\mu\text{g}/\text{dl}$, much lower than the levels required to induce coma experimentally. This is against the hypothesis that hyperammonemia alone is sufficient to produce the encephalopathy found in Reye's syndrome. It is possible that ammonia is one of the toxins but not the sole cause.

A synergistic effect between ammonia and short-chain fatty acids in production of coma in experimental animals is documented.²⁸ Since short-chain fatty acids are elevated in Reye's syndrome, it is possible that they are related to simultaneous elevations of ammonia and fatty acids in these patients.

ROLE OF SHORT-CHAIN FATTY ACIDS

Various short-chain fatty acids (propionate, valerate, isovalerate, and octanoate) are elevated in the serum of patients with Reye's syndrome during the acute phase of the illness. The cause of the elevation is not known, although it is hypothesized that inhibition of mitochondrial beta oxidation that results in the accumulation of these fatty acids. In addition to Reye's syndrome there are other conditions that have common clinical features of encephalopathy and have in common elevated concentrations of short-chain fatty acids or of specific short-chain fatty acids. These include hepatic encephalopathy, Jamaican vomiting syndrome, and isovaleric acidemia.

Numerous experimental studies have shown that short-chain fatty acids are toxic to the central nervous system. When injected into experimental animals, they produce coma, hyperventilation, and EEG changes.²⁹ In addition, fatty acids inhibit liver mitochondria in vitro and inhibit mitochondrial respiration. Fatty acid injection blocks the Krebs cycle and results in a secondary hypercapnic respiratory acidosis. Intracranial pressure elevations have been observed in animals given short-chain fatty acid infusion.

These and other experimental studies suggest that short-chain fatty acids are toxic to the central nervous system and are capable of producing symptoms similar to those of Reye's syndrome. It is likely that these fatty acids are endogenous toxins that either alone or in combination with other toxins such as ammonia result in the encephalopathy found in Reye's syndrome.

children, in particular those symptoms or varicella infection.

As a basis, an attempt was made to re-interpret the results by simultaneous exposure to both agents. This experiment failed to demonstrate the production of Reye's syndrome. Animals were infected with influenza A/Hong Kong or influenza B and given intragastric feedings of large doses of salicylic acid. These treatments produced some depression of aspartate aminotransferase activity and mild fatty changes in the liver but did not potentiate the effects of the virus. These changes are similar enough to those found in human patients to constitute an experimental model for the disease but do not necessarily rule out a role for salicylic acid as one of the human disease, however.

Salicylic acid, a low molecular weight, heat-labile toxin, isolated from serum of patients with Reye's syndrome, inhibits mitochondrial respiration. There is some controversy about the role of salicylic acid. While the work of Gillette with rat liver mitochondria suggests that salicylic acid is the toxic agent whereas Ansevin²⁷ suggests that short-chain fatty acids are the serum factors, at least in brain mitochondria. Although serum fatty acid concentrations are elevated in patients with Reye's syndrome, it is possible that this substance plays a significant role in the disease. On the other hand, short-chain fatty acids, elevated in Reye's syndrome, have been shown to cause mitochondrial swelling in vitro and to inhibit mitochondrial respiration activity. These agents may well be the

present in virtually all patients with Reye's syndrome during the course of the illness. Several investigators have found a correlation between the degree of hyperammonemia and the outcome of the disease, although this is not uniform. Elevations of serum ammonia in experimental animals are capable of producing an endogenous toxin that might be related to the encephalopathy in Reye's syndrome.

Against the hypothesis that ammonia is the toxic agent, the first is that serum ammonia concentrations return to normal within 24-48 hours of the onset of the illness, whereas clinical symptoms may continue. A second is that ammonia infusion into experimental animals requires extremely high serum concentrations (1,000 $\mu\text{g/dl}$) before coma is induced.²⁸ In

normal human subjects, blood ammonia concentrations can be raised to greater than 700 $\mu\text{g/dl}$ without encephalopathic changes. Although concentrations of ammonia found in patients with Reye's syndrome vary widely, they often are in the range of 150-500 $\mu\text{g/dl}$, much lower than the concentrations required to induce coma experimentally. These observations argue against the hypothesis that hyperammonemia alone is sufficient to produce the encephalopathy found in Reye's syndrome. It is possible that ammonia is one of the toxins responsible for the coma but not the sole cause.

A synergistic effect between ammonia and short-chain fatty acids in production of coma in experimental animals has been documented.²⁸ Since short-chain fatty acidemia is also a feature of Reye's syndrome, it is possible that the encephalopathy is related to simultaneous elevations of ammonia and short-chain fatty acids in these patients.

ROLE OF SHORT-CHAIN FATTY ACIDS

Various short-chain fatty acids (propionate, butyrate, isobutyrate, valerate, isovalerate, and octanoate) are consistently elevated in the serum of patients with Reye's syndrome during the acute phase of the illness. The cause of this organic acidemia is not known, although it is hypothesized that there is a block in mitochondrial beta oxidation that results in fatty acidemia.¹⁵ In addition to Reye's syndrome there are several other diseases that have common clinical features of coma and hyperventilation and have in common elevated concentrations of serum free fatty acids or of specific short-chain fatty acids. These include hepatic encephalopathy, Jamaican vomiting sickness, Udorn encephalopathy, and isovaleric acidemia.

Numerous experimental studies have demonstrated that short-chain fatty acids are toxic to the central nervous system. When injected into experimental animals, they are capable of producing coma, hyperventilation, seizures, and electroencephalographic changes.²⁹ In addition, fatty acids produce swelling of liver mitochondria in vitro and inhibit mitochondrial respiration. Fatty acid injection blocks the normal function of the urea cycle and results in a secondary hyperammonemia. Recently, intracranial pressure elevations have been documented during short-chain fatty acid infusion.

These and other experimental studies suggest that short-chain fatty acids are toxic to the central nervous system and are capable of producing symptomatology similar to that found in Reye's syndrome. It is likely that these agents also serve as endogenous toxins that either alone or in combination with other toxins such as ammonia result in the encephalopathic changes found in Reye's syndrome.

REYE'S SYNDROME AS A MITOCHONDRIAL DISORDER

Partin et al.¹³ have emphasized that the primary pathologic change in Reye's syndrome is in the mitochondria, which are swollen, pleiomorphic, and fragmented. DeVivo³⁰ suggests that all of the metabolic abnormalities found in Reye's syndrome can be explained on the basis of impaired mitochondrial function. Activities of several mitochondrial enzymes, including ornithine transcarbamoylase, carbamyl phosphate synthetase, citrate synthetase, glutamic dehydrogenase, succinic dehydrogenase, pyruvate decarboxylase, and pyruvate dehydrogenase, are all depressed in biopsy or autopsy specimens of liver whereas activities of various cytoplasmic enzymes (glucose-6-phosphatase, fructose-1,6-diphosphatase) are within the normal range.

The argument for Reye's syndrome as a primary mitochondrial disorder certainly would seem reasonable given the above evidence. The cause of the mitochondrial dysfunction, however, remains to be elucidated.

EXPERIMENTAL MODELS OF REYE'S SYNDROME

An experimental model for a disease should meet the criteria of reproducing in the animal the principal clinical, biochemical, and pathologic features of the human disease. For Reye's syndrome, the primary clinical features include coma, seizures, hyperventilation, and increased intracranial pressure. The biochemical abnormalities include hyperammonemia, short-chain fatty acidemia, lactic acidemia, hyperaminoacidemia, elevations in serum transaminases, prolonged prothrombin time, increased brain concentrations of octopamine, decreased brain concentrations of dopamine and norepinephrine, inhibition of mitochondria urea cycle enzymes, and hypoglycemia. Pathologic abnormalities include microvesicular fatty accumulation in the liver, distortion and swelling of liver mitochondria, dilatation of the smooth endoplasmic reticulum, and cerebral edema.

Several animal models have been proposed that meet some or most of these criteria. The first one utilized continuous infusion of the short-chain fatty acid sodium octanoate intravenously into rabbits and rats. Such an infusion produces clinical changes, including coma, seizures, hyperventilation, and electroencephalographic slowing, and biochemical abnormalities, including hyperammonemia, hypoglycemia, elevations in serum transaminases, lactic acidemia, and increased brain concentrations of octopamine. Pathologic changes in the animal model include microvesicular fatty accumulation in liver and distortion and swelling of liver mitochondria.

Intraperitoneal injection of 4-pentenoic acid, an analogue of hypoglycine A (believed to be the cause of Jamaican vomiting

sickness), produces coma, seizures, in rats. Fasted rats develop hypoglycemia, ammonia, serum glutamic oxaloacetic transaminase (SGOT) and blood urea nitrogen (BUN) levels. The histology consists of microvesicular fatty accumulation in hepatocytes.

Three other models have utilized the same model. In one, mice were exposed to a fatal encephalomyocarditis virus with mouse encephalomyocarditis virus. In another, a fatal encephalopathy with seizures was demonstrated in mice. In a third, a fatal encephalopathy with seizures was demonstrated in mice. In a fourth, a fatal encephalopathy with seizures was demonstrated in mice. In a fifth, a fatal encephalopathy with seizures was demonstrated in mice. In a sixth, a fatal encephalopathy with seizures was demonstrated in mice. In a seventh, a fatal encephalopathy with seizures was demonstrated in mice. In an eighth, a fatal encephalopathy with seizures was demonstrated in mice. In a ninth, a fatal encephalopathy with seizures was demonstrated in mice. In a tenth, a fatal encephalopathy with seizures was demonstrated in mice. In an eleventh, a fatal encephalopathy with seizures was demonstrated in mice. In a twelfth, a fatal encephalopathy with seizures was demonstrated in mice. 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In a seventy-third, a fatal encephalopathy with seizures was demonstrated in mice. In a seventy-fourth, a fatal encephalopathy with seizures was demonstrated in mice. In a seventy-fifth, a fatal encephalopathy with seizures was demonstrated in mice. In a seventy-sixth, a fatal encephalopathy with seizures was demonstrated in mice. In a seventy-seventh, a fatal encephalopathy with seizures was demonstrated in mice. In a seventy-eighth, a fatal encephalopathy with seizures was demonstrated in mice. In a seventy-ninth, a fatal encephalopathy with seizures was demonstrated in mice. In an eightieth, a fatal encephalopathy with seizures was demonstrated in mice. In an eighty-first, a fatal encephalopathy with seizures was demonstrated in mice. In an eighty-second, a fatal encephalopathy with seizures was demonstrated in mice. In an eighty-third, a fatal encephalopathy with seizures was demonstrated in mice. In an eighty-fourth, a fatal encephalopathy with seizures was demonstrated in mice. In an eighty-fifth, a fatal encephalopathy with seizures was demonstrated in mice. In an eighty-sixth, a fatal encephalopathy with seizures was demonstrated in mice. In an eighty-seventh, a fatal encephalopathy with seizures was demonstrated in mice. In an eighty-eighth, a fatal encephalopathy with seizures was demonstrated in mice. In an eighty-ninth, a fatal encephalopathy with seizures was demonstrated in mice. In a ninetieth, a fatal encephalopathy with seizures was demonstrated in mice. In a ninety-first, a fatal encephalopathy with seizures was demonstrated in mice. In a ninety-second, a fatal encephalopathy with seizures was demonstrated in mice. In a ninety-third, a fatal encephalopathy with seizures was demonstrated in mice. In a ninety-fourth, a fatal encephalopathy with seizures was demonstrated in mice. In a ninety-fifth, a fatal encephalopathy with seizures was demonstrated in mice. In a ninety-sixth, a fatal encephalopathy with seizures was demonstrated in mice. In a ninety-seventh, a fatal encephalopathy with seizures was demonstrated in mice. In a ninety-eighth, a fatal encephalopathy with seizures was demonstrated in mice. In a ninety-ninth, a fatal encephalopathy with seizures was demonstrated in mice. In a hundredth, a fatal encephalopathy with seizures was demonstrated in mice.

One or more of these models may be useful in the pathogenesis of Reye's syndrome and effective treatments for the disease.

OTHER DISEASES SIMILAR TO REYE'S SYNDROME

JAMAICAN VOMITING SICKNESS

This disorder is indigenous to the Caribbean. Clinical symptoms include rapid onset of lethargy, delirium, and vomiting. The symptoms consist of severe hypoglycemia, hyperammonemia, and elevations in serum transaminases. Pathologic changes include microvesicular fatty accumulation in the liver and cerebral edema. The disorder is caused by ingestion of the unripe ackee fruit, *Blaugia aximin*, which contains hypoglycine A, a plant toxin. When ingested, hypoglycine A produces similar symptoms to those found in Reye's syndrome, including hypoglycemia, hyperammonemia, and elevation in short-chain fatty acids.

UDORN ENCEPHALOPATHY

The clinical, biochemical, and pathologic changes in Udorn encephalopathy are similar to those found in Reye's syndrome. The disorder is thought to be caused by ingestion of the plant toxin B1.

CHONDRIAL DISORDER

asized that the primary pathologic is in the mitochondria, which are re-nted. DeVivo³⁰ suggests that li- found in Reye's syndrome can f impaired mitochondrial function. idrial enzymes, including ornithine phosphate synthetase, citrate syn-ase, succinic dehydrogenase, pyru-vate dehydrogenase, are all de-psy specimens of liver whereas smic enzymes (glucose-6-phospha-se) are within the normal range. syndrome as a primary mitochon-l seem reasonable given the above itochondrial dysfunction, however,

F REYE'S SYNDROME

a disease should meet the criteria the principal clinical, biochemical, e human disease. For Reye's syn-eatures include coma, seizures, hy-d intracranial pressure. The bio-de hyperammonemia, short-chain a, hyperaminoacidemia, elevations onged prothrombin time, increased amine, decreased brain concentra-inephrine, inhibition of mitochon-l hypoglycemia. Pathologic abnor-a-ty accumulation in the liver, e. tochondria, dilatation of the n, and cerebral edema.

e been proposed that meet some or st one utilized continuous infusion xidium octanoate intravenously into ision produces clinical changes, in-erventilation, and electroenceph-ochemical abnormalities, includ-oglycemia, elevations in serum ia, and increased brain concentra-ic changes in the animal model in-cumulation in liver and distortion ndria.

f 4-pentenoic acid, an analogue of e the cause of Jamaican vomiting

sickness), produces coma, seizures, hyperventilation, and death in rats. Fasted rats develop hypoglycemia; elevations in serum ammonia, serum glutamic oxaloacetic transaminase (SGOT), and blood urea nitrogen (BUN) have been documented. Liver histology consists of microvesicular fatty accumulation in hepatocytes.

Three other models have utilized viruses to simulate this syndrome. In one, mice were exposed to insecticides and inoculated with mouse encephalomyocarditis virus. The animals developed a fatal encephalopathy with seizures; liver and kidney histology demonstrated fatty accumulation. In the second model, Mengo virus was found to potentiate the effect of 4-pentenoic acid in producing encephalopathy, hypoglycemia, and fatty accumulation in the viscera of rats. The third viral model utilized influenza B/Lee virus inoculated into four-week-old mice. This agent produced coma, seizures, elevated transaminases, inhibition of the urea cycle enzymes ornithine transcarbamoylase and carbamyl phosphate synthetase, and fatty infiltration of the liver.

One or more of these models may prove useful in studying the pathogenesis of Reye's syndrome and in finding more specific and effective treatments for the disease.

OTHER DISEASES SIMILAR TO REYE'S SYNDROME

JAMAICAN VOMITING SICKNESS

This disorder is indigenous to Jamaica and is limited to childhood. Clinical symptoms include repeated vomiting progressing rapidly to lethargy, delirium, and coma. Biochemical abnormalities consist of severe hypoglycemia and abnormalities of liver function. Pathologic changes include diffuse fatty accumulation in the liver and cerebral edema. This disorder is thought to be due to ingestion of the unripe ackee fruit, which contains hypoglycine A, a plant toxin. When injected into animals, hypoglycine A produces similar symptomatology and also causes an elevation in short-chain fatty acid levels.

UDORN ENCEPHALOPATHY

The clinical, biochemical, and pathologic features are identical to those found in Reye's syndrome. However, Udorn encephalopathy is thought to be caused by ingestion of a fungal toxin, aflatoxin B1.

ISOVALERIC ACIDEMIA

This disorder is an inborn error of organic acid metabolism involving a defect in the activity of the enzyme isovaleryl CoA dehydrogenase. Symptoms in these children may be episodic and, especially when given a leucine load, they develop vomiting, lethargy, and coma, with hypoglycemia and abnormalities in liver function. These episodes tend to be recurrent and may lead to mental retardation.

HEPATIC ENCEPHALOPATHY CAUSED BY TOXIC OR VIRAL HEPATITIS, CIRRHOSIS, AND OTHER ETIOLOGIES

Hepatic encephalopathy produces a syndrome of lethargy, delirium, and coma with a variety of metabolic abnormalities, including abnormal liver function tests with hyperammonemia, hyperbilirubinemia, hypoglycemia, and short-chain fatty acidemia. The liver pathology in hepatic coma is quite different from that in Reye's syndrome, showing extensive hepatic cellular necrosis and inflammatory changes as well as fatty accumulation. Patients with hepatic encephalopathy also develop cerebral edema.

SYSTEMIC CARNITINE DEFICIENCY

Systemic carnitine deficiency is a metabolic disorder that at times resembles Reye's syndrome.³¹ Although patients with this disorder tend to have a slowly progressive myopathy, they may develop acute and recurrent episodes of lethargy or coma, with seizures, hypoglycemia, and abnormal liver function tests. Liver biopsy may demonstrate fatty accumulation. Serum carnitine levels may be normal or depressed; thus, tissue carnitine determinations are necessary to make the diagnosis.

TREATMENT OF REYE'S SYNDROME

Since the etiology of Reye's syndrome is not known, treatment is of necessity aimed at the symptomatology rather than the cause.

In Reye's original paper he reported on several attempts at treatment. These included dexamethasone, high concentrations of glucose, and a combination of glucose and insulin. Each of these was tried in a small number of patients and appeared to make no difference in survival. The first major contribution to a therapeutic approach came in 1972, when Huttenlocher³² reported improved survival rates using exchange transfusion. The rationale for exchange transfusion was threefold: to clear the hyperammonemia, to replace coagulation factors, and to remove

"unknown toxins." Since then, continued with exchange transfusion, a difference in survival between patients receiving transfusion and those treated with glucose alone whereas others have found that exchange transfusion proved survival significantly.

High-dose glucose and insulin therapy has been used as an adjunct to the management of Reye's syndrome based on the fact that free fatty acids are elevated in the serum of patients with the syndrome. Fatty acids block fatty acid release from adipose tissue by the enzyme lipoprotein lipase, and glucose inhibits the reaction such that fatty acids are not released from adipose tissue. This form of therapy has been used but its efficacy is difficult to assess because of the adjunct to other therapy that the patient receives.

Peritoneal dialysis has been used to remove toxins from the body and initially showed promise, but subsequent experience shows that it is not effective from this approach. In the past few years, monitoring has become a popular part of the regimen. More aggressive attempts at treatment have evolved and will be discussed.

A general outline to the treatment of Reye's syndrome is divided into three phases: intensive supportive care, treatment of metabolic abnormalities, and treatment of cerebral pressure.

INTENSIVE SUPPORTIVE CARE

Regardless of the etiology of Reye's syndrome, treatment approaches are universal. Patients should be treated in a pediatric intensive care unit. Care should be carried out as soon as the patient is admitted to the staging system developed by the Consensus Development Conference on the Management of Reye's Syndrome in March 1975 for uniform use around the country. The treatment has been outlined in Table 5.

For a child in stage I or II coma, treatment includes frequent neurologic evaluation (with a glucose solution), and correction of electrolyte imbalances. For children who are in stage III or IV coma, treatment is a composite of several approaches. The program is currently used at the University of California at San Diego.³³

of organic acid metabolism of the enzyme isovaleryl CoA. In children, the disease may be episodic. In the adult, they develop vomiting, hypoglycemia and abnormalities tend to be recurrent and may

DIFFERENTIAL DIAGNOSIS BY TOXIC OR VIRAL ETIOLOGIES

Reye's syndrome is a syndrome of lethargy, decreased consciousness, metabolic abnormalities, increased ammonia, and short-chain fatty acids. Coma is quite different from extensive hepatic cellular necrosis as well as fatty accumulation. Pathology also develops cerebral

Reye's is a metabolic disorder that at onset. Although patients with this aggressive myopathy, they may have signs of lethargy or coma, with abnormal liver function tests. Liver biopsy is abnormal. Serum carnitine levels are low. Tissue carnitine deficiency is characteristic of the disease.

PROGNOSIS

Prognosis is not known, treatment is supportive rather than the

based on several attempts at treatment. High concentrations of glucose and insulin. Each of these treatments appeared to be a first major contribution to a cure. In 1972, when Huttenlocher³² reported exchange transfusion. The treatment was threefold: to clear the blood of toxins, and to remove

"unknown toxins." Since then, conflicting results have been obtained with exchange transfusion, some centers reporting no difference in survival between patients treated with exchange transfusion and those treated with intensive supportive therapy alone whereas others have found that exchange transfusion improved survival significantly.

High-dose glucose and insulin therapy was suggested in 1972 as an adjunct to the management of patients with Reye's syndrome based on the fact that free fatty acid concentrations were elevated in the serum of patients with this disease. Insulin blocks fatty acid release from adipose tissue by inhibition of the enzyme lipoprotein lipase, and glucose reverses the equilibrium of the reaction such that fatty acids are transported back into adipose tissue. This form of therapy has been used sporadically, but its efficacy is difficult to assess because it is used as an adjunct to other therapy that the patient is receiving.

Peritoneal dialysis has been used to clear ammonia and other toxins from the body and initially met with good success. However, subsequent experience showed little additional benefit from this approach. In the past few years, intracranial pressure monitoring has become a popular addition to the therapeutic regimen. More aggressive attempts to control intracranial pressure have evolved and will be discussed below.

A general outline to the treatment of Reye's syndrome can be divided into three phases: intensive supportive care, correction of metabolic abnormalities, and treatment of elevated intracranial pressure.

INTENSIVE SUPPORTIVE CARE

Regardless of the etiology of Reye's syndrome, certain basic treatment approaches are universally followed. All children with a diagnosis of Reye's syndrome should be admitted to a pediatric intensive care unit. Clinical staging of the disease should be carried out as soon as the diagnosis is suspected. The staging system developed by the National Institutes of Health Consensus Development Conference on the Diagnosis and Treatment of Reye's Syndrome in March of 1981 is being suggested for uniform use around the country. This staging system has been outlined in Table 5.

For a child in stage I or II coma, supportive care should consist of frequent neurologic evaluations, hypertonic glucose (15% dextrose solution), and correction of any electrolyte abnormalities. For children who are in stage III or below there are several variations of intensive care used in different centers. The following is a composite of several approaches and is the protocol currently used at the University of California Medical Center in San Diego.³³

Arterial and central venous pressure lines are inserted, as are nasogastric tubes and Foley catheters. Strict intake and output are recorded. Intravenous solutions consisting of 20% glucose and a balanced electrolyte solution are given initially at two-thirds maintenance. The patients are electively intubated with an endotracheal tube and mechanical ventilation instituted as needed. Temperature is kept at normal levels through the use of a cooling mattress if necessary. Methylcellulose eyedrops are administered to both eyes every four hours. Any unnecessary sensory stimulation of the patient is avoided.

CORRECTION OF METABOLIC ABNORMALITIES

Serum glucose concentrations are kept between 125 and 175 mg/dl and serum osmolality below 310 milliosmols. Intravenous fluid solutions are increased or decreased as necessary to maintain the serum osmolality in an acceptable range.

The role of chest physiotherapy in patients with Reye's syndrome is controversial. Although excessive stimulation of the patient may increase intracranial pressure, accumulation of mucus plugs in the bronchial tree may increase intrathoracic pressure, which, in turn, will also increase intracranial pressure. I advocate careful chest physiotherapy using a vibrator followed by brief suctioning to be performed every two hours. The patient is not turned for postural drainage but is kept in the supine position with the head of the bed at a 30-degree elevation.

Insulin, 1 unit per 5 gm of glucose given, is administered intravenously every four hours. As stated previously, the rationale for this is to attempt to decrease serum free fatty acid concentrations by stimulating the enzyme lipoprotein lipase. If insulin is used, serum glucose concentrations or dextrostix are checked immediately before and one-half hour after the insulin dose. If the glucose concentration is greater than 175 mg/dl, the amount of insulin is increased; if less than 125 mg/dl, the amount of glucose is increased.

Neomycin by nasogastric tube at a dose of 100 mg/kg/day and/or lactulose 1-2 gm/kg/day by nasogastric tube may be used to reduce ammonia accumulation. Prothrombin time and other coagulation abnormalities may be corrected by administration of fresh-frozen plasma 10 ml/kg or by a double volume exchange transfusion. This mode of therapy will also reduce serum ammonia concentrations and should be considered in particular in patients with a serum ammonia concentration of greater than 350 μ g/dl. Although not proved to be effective, vitamin K 1-5 mg usually is administered daily until clotting studies improve.

If clinical seizures occur, or electroencephalographic evidence of seizure activity is present, intravenous phenytoin 5-7 mg/kg/day after a loading dose of 15-20 mg/kg may be used for seizure

control. Barbiturates should be avoided to alter the neurologic examination. Lidocaine and other sedatives should be avoided in the venous and arterial lines should be used because heparin stimulates fatty acid

TREATMENT OF ELEVATED INTRACRANIAL PRESSURE

Once the prothrombin time is corrected, a pressure monitor should be inserted. The monitor may be inserted into the ventricular, subarachnoid, or epidural space, depending on the preference of the neurosurgeon. The data of several other centers suggest that epidural monitors are more accurate than other monitors. Epidural monitors are greater risks of infection and bleeding. Intracranial pressure should be monitored continually and preferably with a polygraph using a pressure transducer. Intracranial pressure greater than 20 mm Hg is a contraindication to intracranial pressure monitoring. Intracranial pressure monitoring is of cerebral perfusion. Cerebral perfusion pressure is the mean arterial blood pressure minus intracranial pressure. When cerebral perfusion pressure falls below 50 mm Hg, cerebral ischemia may occur; at levels less than 40 mm Hg, irreversible brain damage may occur. To maintain cerebral perfusion pressure, intracranial pressure should be kept below 20 mm Hg.

The treatment of intracranial pressure includes the following measures.

1. Mannitol 0.25 gm/kg per dose. Mannitol should be used to keep intracranial pressure below 20 mm Hg. The maximum dose of mannitol is 0.5 gm/kg. Serum osmolality with potential for cerebral edema. Serum osmolality is kept below 310 mOsm/kg. Mannitol continues to be effective and there is no evidence of toxicity in this form of therapy. The primary concern is hyperosmolality, which, in turn, may lead to dehydration, hypotension, and "mannitol toxicity." Mannitol should be avoided by adequate fluid replacement.
2. Controlled hyperventilation. Hyperventilation to a PCO_2 of 25-30 torr.
3. Muscle paralysis with pancuronium bromide 0.1 mg/kg per dose.
4. If an intraventricular catheter is inserted, cerebrospinal fluid can be released. Release of more than 1-1.5 ml of cerebrospinal fluid since the ventricles are small. Release of fluid may cause ventricular collapse. A pressure device.

pressure lines are inserted, as are catheters. Strict intake and output measurements consisting of 20% glucose solution are given initially at two-hour intervals. The patient is selectively intubated with an endotracheal tube and mechanical ventilation instituted as needed to maintain normal levels through the use of methylcellulose eyedrops are administered every two hours. Any unnecessary sensory stimulation is avoided.

ABNORMALITIES

Arterial pressures are kept between 125 and 175 mm Hg and serum osmolality below 310 milliosmols. Intravenous fluids are increased as necessary to maintain an acceptable range.

In patients with Reye's syndrome, excessive stimulation of the respiratory system, increase in intracranial pressure, accumulation of mucus, may increase intrathoracic pressure and increase intracranial pressure. Therapy using a vibrator followed by suction every two hours. The patient is kept in the supine position but is kept in the supine position at a 30-degree elevation.

Insulin given, is administered intravenously if not previously, the rationale being to free fatty acid concentration and free fatty acid lipase. If insulin is given and dextrostix are checked every two hours after the insulin dose. If serum glucose is 175 mg/dl, the amount of insulin is 0.25 mg/kg/day, the amount of

at a dose of 100 mg/kg/day and/or nasogastric tube may be used to correct prothrombin time and other coagulation factors by administration of vitamin K. It will also reduce serum ammonia and be considered in particular in patients with a concentration of greater than 10 mg/dl. If effective, vitamin K 1-5 mg/kg/day until clotting studies improve. Electroencephalographic evidence of venous phenytoin 5-7 mg/kg/day may be used for seizure

control. Barbiturates should be avoided if possible, as they will alter the neurologic examination. Likewise, Valium, morphine, and other sedatives should be avoided where possible. Heparin in the venous and arterial lines should be kept to a minimum because heparin stimulates fatty acid release.

TREATMENT OF ELEVATED INTRACRANIAL PRESSURE

Once the prothrombin time is corrected, an intracranial pressure monitor should be inserted. The choice of monitor (intraventricular, subarachnoid, or epidural) will depend to a large extent on the preference of the neurosurgeon. My experience and that of several other centers suggests that intraventricular catheters are more accurate than other types but also carry slightly greater risks of infection and bleeding. Intracranial pressure should be monitored continually until the patient awakens, preferably with a polygraph using a paper tracing. An intracranial pressure greater than 20 mm Hg should be treated. The goal of intracranial pressure monitoring is to prevent any compromise of cerebral perfusion. Cerebral perfusion pressure is equal to the mean arterial blood pressure minus the intracranial pressure. When cerebral perfusion pressure drops below 50, cerebral ischemia may occur; at levels less than 30, neuronal death occurs, with irreversible brain damage. Thus, the immediate goal is to maintain cerebral perfusion pressure above 50 mm Hg.

The treatment of intracranial pressure elevations consists of the following measures.

1. Mannitol 0.25 gm/kg per dose intravenously as needed to keep intracranial pressure below 20 mm Hg. There is no absolute maximal dose of mannitol; the dose-limiting factor is hyperosmolality with potential compromise of renal function. If serum osmolality is kept below 310 milliosmols, mannitol continues to be effective and there is less risk of complication from this form of therapy. The primary complication of osmotherapy is hyperosmolality, which, in turn, leads to intravascular depletion, hypotension, and "mannitol nephrosis." All of these can be avoided by adequate fluid replacement.

2. Controlled hyperventilation with a mechanical respirator to a PCO_2 of 25-30 torr.

3. Muscle paralysis with pancuronium bromide 0.1-0.2 mg/kg per dose.

4. If an intraventricular catheter is used, small amounts of cerebrospinal fluid can be released through this for immediate control of intracranial pressure. Care must be taken to prevent release of more than 1/2-1 ml of cerebrospinal fluid each time, since the ventricles are small and release of large amounts of fluid may cause ventricular collapse and malfunction of the pressure device.

5. Careful chest physiotherapy to remove mucus plugs results in better control of intracranial pressure.

The use of high-dose barbiturates has been advocated in the treatment of intracranial pressure elevations in Reye's syndrome.³⁴ Barbiturates are thought to decrease cerebral metabolic demands, decrease cerebral blood flow, and thus control intracranial pressure. This form of therapy has been used with success in the treatment of closed head trauma. Several centers have reported excellent results with control of intracranial pressure in Reye's syndrome using high-dose barbiturates. However, survival rates vary considerably with this form of therapy. Some groups have reported increased survival rates whereas others find no difference in survival between patients managed with osmotherapy and those in whom barbiturate coma has been used. Since high-dose barbiturates may cause added complications of hypotension, hypoxia, and changes in cardiac output, their use should not be undertaken lightly. This treatment should be reserved for cases in which intracranial hypertension is refractory to other modes of therapy.

The usual method for inducing barbiturate "coma" is the following: Pentobarbital is administered intravenously in an initial bolus of 3-4 mg/kg followed by doses of 100-200 mg per hour until a blood level of 3-4 mg/dl is reached. This level is maintained until brain compliance improves and intracranial pressure drops to normal or unless complications of treatment occur. Pentobarbital should be tapered but not stopped abruptly. Complications include a drop in arterial blood pressure, unexplained change in cardiac output, or unexplained hypoxia. Once this treatment is initiated, clinical assessment is impossible because of iatrogenically induced coma. Thus, sophisticated monitoring devices are necessary so that cardiac output and central venous pressure can be measured accurately. Complete ventilatory control is necessary and frequent arterial blood gas determinations are needed.

Decompressive craniectomy is used at some centers for treatment of refractory intracranial pressure elevations. Some success has been reported using this technique. Such a procedure should be reserved for the most difficult cases, since many potential risks are involved, including infection and bleeding.

Hypothermia as a mechanism for lowering intracranial pressure has not been shown to improve survival thus far in Reye's syndrome. It is unlikely that control of intracranial pressure alone will insure a good outcome in patients with Reye's syndrome, since there are multiple metabolic abnormalities associated with the disease. A reasonable approach to therapy would be to control intracranial pressure while at the same time correcting hypoglycemia, hyperammonemia, and clotting abnormalities as suggested above.

PROGNOSIS

The prognosis for improved survival of survival has improved greatly. Whereas mortality rate previously was between 10% and 40% using variation therapy and intracranial pressure improved survival rate, an effort has been made to reduce the neurologic sequelae in survivors of Reye's syndrome. 10% of survivors are left severely brain damaged. Recent reports³⁵ suggest that virtually all survivors of Reye's syndrome over the age of two years turned to grossly normal functioning. When neurologic testing was performed, some have difficulties with school achievement, sequencing, tactile problem solving. These deficits appeared to correlate with the severity and the length of coma.

Of importance is the fact that the neurologic episode may take months to years to resolve within a few days to weeks. The neurologic function, memory, or language ability may take months. Therefore, it is necessary to wait several years before ultimate outcome can be determined. In children tested two years or more after the episode, very subtle neuropsychologic deficits may be reflected in some difficulty with learning enough to impair overall function.

With early diagnosis of the syndrome and treatment before stage III coma supervenes, both survival rate and absence of sequelae is excellent.

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to remove mucus plugs results in pressure.

Barbiturates have been advocated in the presence of pressure elevations in Reye's syndrome to decrease cerebral metabolic flow, and thus control intracranial pressure. This therapy has been used with success in head trauma. Several centers have reported success with control of intracranial pressure with high-dose barbiturates. However, there is controversy with this form of therapy. Some studies show improved survival rates whereas others show no difference between patients managed with barbiturates and those with normobarbiturate coma. Barbiturate coma has been associated with complications such as hypotension, decreased cardiac output, and changes in cardiac output, which may be overlooked. This treatment, which controls intracranial hypertension, is a supportive therapy.

Normobarbiturate "coma" is the following: barbiturate is administered intravenously in an initial dose of 100-200 mg per kg. A level of 4 mg/dl is reached. This level is maintained. Once pressure improves and intracranial pressure is controlled, complications of treatment are less. Barbiturates are tapered but not stopped abruptly. In the presence of arterial blood pressure, unexplained hypoxia, or unexplained hypoxia. Once a clinical assessment is impossible because of coma. Thus, sophisticated monitoring that cardiac output and central venous pressure are not monitored accurately. Complete ventilation and arterial blood gas determination.

is used at some centers for treatment of pressure elevations. Some studies support this technique. Such a procedure is difficult in difficult cases, since many potential complications such as infection and bleeding. Normobarbiturate for lowering intracranial pressure has improved survival thus far in Reye's syndrome. Control of intracranial pressure is important in patients with Reye's syndrome. The metabolic abnormalities associated with Reye's syndrome are a reasonable approach to therapy would be to control hypoxia while at the same time correct hypoxemia, and clotting abnormalities.

PROGNOSIS

The prognosis for improved survival as well as for better quality of survival has improved greatly over the past few years. Whereas mortality rate previously was 80-100%, it now is between 10% and 40% using variations on intensive supportive therapy and intracranial pressure monitoring. With this improved survival rate, an effort has been made to evaluate neurologic sequelae in survivors of Reye's syndrome. An estimated 10% of survivors are left severely brain damaged. However, recent reports³⁵ suggest that virtually all children who developed Reye's syndrome over the age of two years and survived returned to grossly normal functioning. When specific neuropsychologic testing was performed, some children were found to have difficulties with school achievement, visual motor integration, sequencing, tactile problem solving, and concept formation. These deficits appeared to correlate with the severity of the disease and the length of coma.

Of importance is the fact that children surviving the initial episode may take months to years to recover completely from the neurologic insult. Whereas the overt encephalopathy may resolve within a few days to weeks, subtle deficits in intellectual function, memory, or language abilities may persist for many months. Therefore, it is necessary to follow them for a period of years before ultimate outcome can be assessed accurately. In children tested two years or more after the acute illness, only very subtle neuropsychologic deficits persisted. These deficits may be reflected in some difficulty in learning but are not severe enough to impair overall function.

With early diagnosis of the acute illness and onset of treatment before stage III coma supervenes, the prognosis in terms of both survival rate and absence of long-term neurologic sequelae is excellent.

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SELF-ASSESSMENT ANSWERS

- | | |
|------|-------|
| 1. d | 6. c |
| 2. b | 7. d |
| 3. b | 8. b |
| 4. c | 9. d |
| 5. d | 10. c |

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SELF-ASSESSMENT ANSWERS

- | | |
|------|-------|
| 1. d | 6. c |
| 2. b | 7. d |
| 3. b | 8. b |
| 4. c | 9. d |
| 5. d | 10. c |