PALMER 1979
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SAFETY EVALUATION OF TOOTHPASTE CONTAINING CHLOROFORM II. LONG TERM STUDIES IN RATS

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The results of a preliminary rangefinding 13-wk oral toxicity study and of two longer term studies on chloroform in toothpaste base are reported. Significant changes in serum enzymes and certain haemotological parameters were seen at the higher dose-levels in the rangefinding study. Intercurrent disease made it necessary to terminate the first long-term experiment prematurely after I yr. No evidence of serious toxicity was recorded. In the second long-term experiment, groups of 50 caesarian-derived SPF Sprague-Dawley rats of each sex received either the equivalent of 60 mg $CHCl_3$/kg/d in toothpaste base or the vehicle only, by gavage on 6 d/wk for 80 wk and were then observed for up to a further 15 wk. Chloroform-treated rats of both sexes survived better than the controls, though both groups had a high incidence of non-neoplastic respiratory and renal disease. Female rats gave a consistent finding of decrease in plasma cholinesterase, shown to be related to activity against butyrylcholine but not acetyl-β-methylcholine.

Tumours of various sites were seen in 39 percent of chloroform-treated rats of both sexes examined histologically, compared with 38 percent of vehicle controls. There were no treatment-related effects on the incidence of liver or kidney tumours. Histologically-malignant mammary tumours were reported in more treated than control rats, but the difference in incidence was not statistically significant.

INTRODUCTION

When this series of studies on toothpaste containing chloroform was being planned, no long-term experiments on rats given chloroform had been reported in the literature. The peculiar susceptibility of some

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Journal of Environmental Pathology and Toxicology 2:821-833

laboratory-bred mouse strains to chloroform-induced nephrotoxicity (Deringer *et al.*, 1953) along with evidence for the unusual morphology of the kidney in such mice (Dunn, 1949) indicated the need for long-term studies in other species. An investigation in the rat involving treatment for 80 wk, to be followed by observation for a further 24 wk, was therefore begun in November 1962. Because of severe respiratory and renal disease in control and treated rats alike, the experiment had to be terminated prematurely after only 1 year. A second study, involving the use of specific pathogen-free rats, was started in January 1966. Respiratory and renal disease killed fewer rats prematurely though the development of mammary tumours reduced the survival of females towards the end of the study. The second study was terminated after 95 wk.

MATERIALS AND METHODS

Selection of Dose-Levels

A preliminary 13-wk study was carried out in Sprague-Dawley rats (10 of each sex per dose level), given daily intragastric doses of toothpaste via a flexible plastic cannula. Treatments in terms of chloroform content were 0, 15, 30, 150 and 410 mg/kg/d. At the highest dose-level, increased liver weight with fatty change and necrosis was recorded. Gonadal atrophy in both sexes and increased cellular proliferation in the bone marrow at 410 mg $CHCl_3$/kg/d indicated definite toxic response. At 150 mg/kg/d there were less pronounced changes, but still a distinct influence on relative liver and kidney weight.

The dose levels chosen for the first long-term study on the basis of the preliminary study findings were equivalent to 15, 75 and 165 mg $CHCl_3$/kg/d in toothpaste base including essential oil flavour components. The chloroform concentration for the top dose-level was adjusted to 10 percent, so that the volume of toothpaste administered each day could be kept to 1 ml/kg. Twenty-five rats of each sex were used for each dose level, and 75 rats of each sex received toothpaste containing neither chloroform nor essential oils.

From the information gathered in the course of the first long-term rat study, treatment for the second study was fixed at a dose-level equivalent to 60 mg $CHCl_3$/kg/d. In other words, treated and control rats each received 1 ml of toothpaste/kg/d, the toothpaste given to the treated group containing 3.5 percent $CHCl_3$. Fifty rats of each sex received toothpaste containing chloroform; the same number of rats received toothpaste without chloroform (but with essential oils).

Animal Management and Treatment

Toothpaste for the rat studies complied with the formula given by Roe *et al.* (1978).

Rats of the Sprague-Dawley strain for the first study came from Animals Suppliers Limited. Caesarian-derived specific pathogen-free Sprague-Dawley rats for the second study were supplied by Carworth

Farms (USA). After initial quarantine and acclimatization for 11-14 days, each rat was weighed and identified by earmark; the rats were housed five to a cage, allocated so as approximately to equalize initial bodyweights between treatment groups. Coprophagy was limited by using suspended cages fitted with wire-mesh flooring. Initial bodyweights were 180-240 g (males) and 130-175 g (females). Spratts Laboratory Animals Diet (autoclaved) and water were provided *ad libitum*. Spatial distribution of cages was arranged to minimize environmental differences between treatments. Animal room temperature was maintained at 21 ± 2°.

In the first study, any rat dying in the initial 8 wk of the investigation was replaced by one of similar bodyweight taken from a reserve stock which had received the same treatment. This replacement procedure was adopted for the first 4 wk only of the second study. The toothpastes were given orally by gavage using a PVC catheter (French gauge No. 3), at the same time each day on 6 d/wk. Care was taken to avoid spillage into the lungs as far as possible.

Observations

Food consumption was recorded; water consumption was noted by inspection but not measured. Each rat was weighed initially and weekly thereafter.

Haematological and biochemical studies were carried out at intervals during the first study along with urinalysis. In view of the minimal changes found in the first study, only serum and erythrocyte cholinesterases were monitored during the second study.

Any rat showing signs of severe debility or intoxication was isolated. If death appeared imminent, blood and urine samples were taken if possible and the rat was then killed. Rats killed *in extremis* or found dead were subjected to detailed macroscopic examination and, where feasible, a full range of tissues was preserved in 4 percent formol-saline.

The following organ-weights were recorded after the rats had been killed by CO_2 euthanasia: adrenals, kidneys, liver, lungs and spleen. Ratio of organ-weight to bodyweight was calculated.

All macroscopically-observed tumours and abnormal growths were presented for histopathological examination along with specimens of brain, lungs, liver and kidneys. Samples representing a wide range of tissues were preserved in 4 percent formol-saline.

RESULTS

Experiment 1

The only recognizable toxic effect was a varying degree of alopecia in many rats at the highest dose level between the 10th and 20th wk but not subsequently. Respiratory disease was evident in all groups after the fifth month and thereafter increased in incidence and severity. Group mean food consumption fluctuated, without significant relationship to treatment. Treatment-related effects on bodyweight were evident only in

males, being minimal at the lowest dose level but significant at the upper dose levels from wk 7 onwards. Body-weight gain of female rats was only slightly depressed at the highest dose level and not affected by the other treatments. From Wk 45 until the experiment was terminated after 52 wk, bodyweights fluctuated and there was marked weight loss in the males of all groups.

Haematological parameters at 16, 32 and 52 wk showed no signifi-

TABLE 1. Terminal (52 week) Data from Experiment I (Sprague-Dawley Rat)

	Males				Females			
Toothpaste: mg $CHCl_3$/kg/d	0	15	75	165	0	15	75	165
No. of rats in group	75	25	25	25	75	25	25	25
No. of survivors	14	4	5	2	48	18	13	13
Terminal Biochemistry: group means								
Blood urea (mg%)	53	46	50	50	51	53	51	44
SGPT (mU/ml)	20	40	26	36	31	24	18	21
SGOT (mU/ml)	107	134	122	115	146	130	125	136
SAP (KA units)	30	39	48	24	26	14	17	13
Plasma Cholinesterase (Δ pH/h)	0.34	0.40	0.34	0.36	0.73	0.46	0.46	0.49
Cholesterol (mg%)	152	108	202	127	101	113	115	139
Serum protein (g%)								
Total	7.1	7.6	7.1	7.4	7.3	7.4	7.5	7.2
Albumin	1.4	1.4	1.5	1.8	2.4	2.6	2.2	2.5
α globulin	2.4	2.3	1.8	1.7	1.8	1.7	2.0	1.6
β globulin	1.6	2.0	1.7	2.5	1.5	1.7	1.6	1.5
γ globulin	1.6	2.2	1.9	1.6	1.6	1.4	1.7	1.5

cant differences between groups. No unusual features of the urinalysis were seen except for a mild degree of aciduria in males at the highest dose level at 32 but not at 52 wk. Table 1 shows the group mean findings for blood chemistry at 52 wk. The main treatment-related findings were increased SGPT and alkaline phosphatase in males and decreased plasma cholinesterase in females (but not males). Cholesterol levels were raised in females at the highest dose level but SAP and SGPT in treated females

were lower than in the controls. However, the group mean values at the end of the study were probably influenced by the relatively small numbers of survivors in the various groups.

Death rates increased rapidly from about the 30th wk of the study. Towards the end of the first year, when the study was terminated, only about 20 percent of males in all groups were still alive. Although post-mortem examination in a few cases indicated that treatment errors had resulted in toothpaste entering the trachea, the majority showed evidence of intercurrent respiratory disease, often with severe consolidation and bronchiectasis.

TABLE 2. Cholinesterase Activity (Δ pH/h) of Rats in Experiment II

	Males				Females			
Toothpaste: mg $CHCl_3$/kg/d	0		60		0		60	
	Plasma	RBC	Plasma	RBC	Plasma	RBC	Plasma	RBC
Week of Expt.								
29	0.45	0.56	0.50	0.53	1.25	0.48	0.83[a]	0.43
34	0.70	0.39	0.71	0.39	1.56	0.33	1.13[a]	0.33
52	0.77	0.56	0.76	0.54	1.86	0.65	1.36[a]	0.61
80	0.72	0.42	0.77	0.43	1.50	0.62	1.21[b]	0.54[a]
95	0.88	0.72	0.83	0.75	1.51	0.61	1.45	0.66

[a]Significant difference from control $p < 0.001$

[b]Significant difference from control $p < 0.01$

Histopathological examination confirmed widespread respiratory and kidney disease in all groups. Special attention was paid to changes in the liver but there was no evidence of severe liver disease in chloroform-treated or control rats. The only neoplastic changes encountered were an undifferentiated malignant tumour of the mediastinum in a high dose-level male rat and a mammary fibroadenoma in a control female.

Experiment II

Toxic effects attributable to treatment were absent except for a marginal, though consistent and progressive, retardation of weight gain in both sexes (Fig. 1). A persistent but small depression of food consumption was noted in treated females compared to the controls, but no such difference was seen in male rats (mean food intake/rat/wk was 157 g for

treated and control males, 119 g for treated and 125 g for control females).

A noteworthy observation during the study was a decrease in plasma but not in erythrocyte cholinesterase in treated female rats only (Table 2). The greatest deviation in actual values was from a group mean of 1.86ΔpH/h at Wk 52 for the control females compared to 1.36ΔpH/h for the treated group. Further investigation showed that female Sprague-Dawley rats exhibited plasma butyrylcholinesterase activity which was inhibited by chloroform treatment, but male rats did not have this enzyme in the blood plasma.

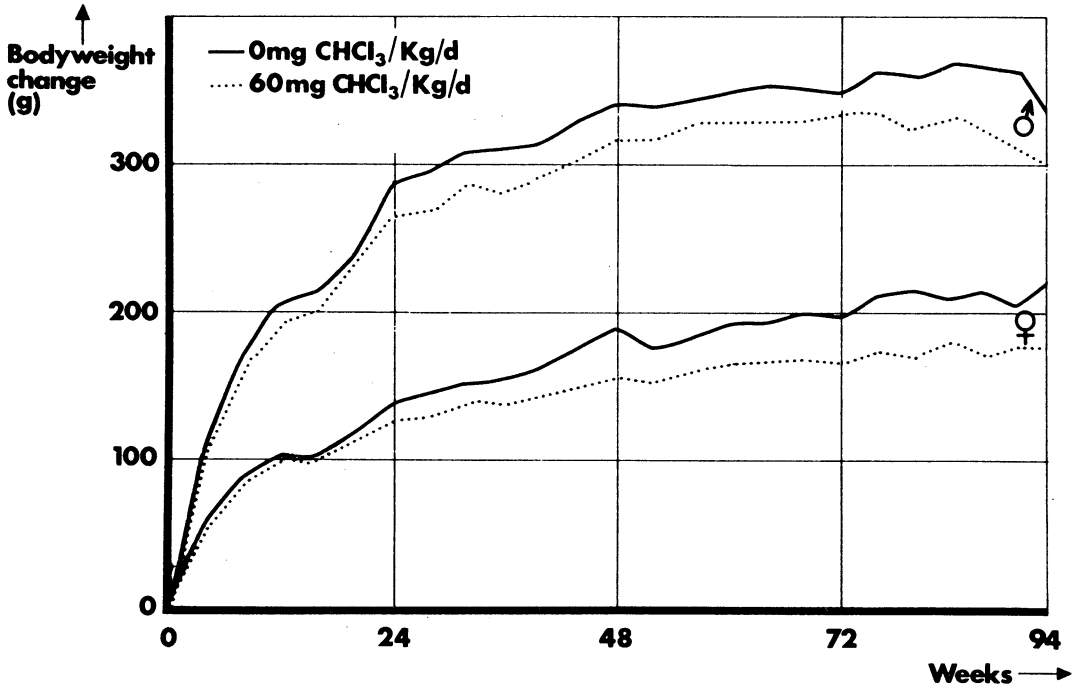

FIGURE 1. Body-weight Changes in Sprague-Dawley Rats (Expt. II)

There were no significant differences in the timing or numbers of deaths during the study between treated and control groups. Thirty-two percent of the treated males survived for 95 wk compared with 22 percent of the control males and 26 percent of the treated females compared with 14 percent of the control females (Fig. 2).

Respiratory disease was encountered in all groups, especially between the 42nd and 56th wk when *Bordetella* infection was identified in animals exhibiting acute respiratory disease. Chronic tubular nephritis with prominent tubular cast formation such as is common in untreated rats of many strains was often found in rats dying late in the study. The main contributory factor to the death rate towards the end of the study

was the need to kill for humane reasons females with large or multiple mammary tumours (14 treated and 13 controls).

The only noteworthy finding in organ-weight analyses (Table 3) was a significant ($p < 0.01$) decrease in relative liver weight for treated female rats. Minor histological changes in the liver were frequently seen, but no severe fatty infiltration, fibrosis or marked bile duct abnormality; there was no evidence of any treatment-related toxic effect in the liver. Qualitatively and quantitatively similar lung disease was apparent in both groups. Slight differences between groups in the incidence of moderately

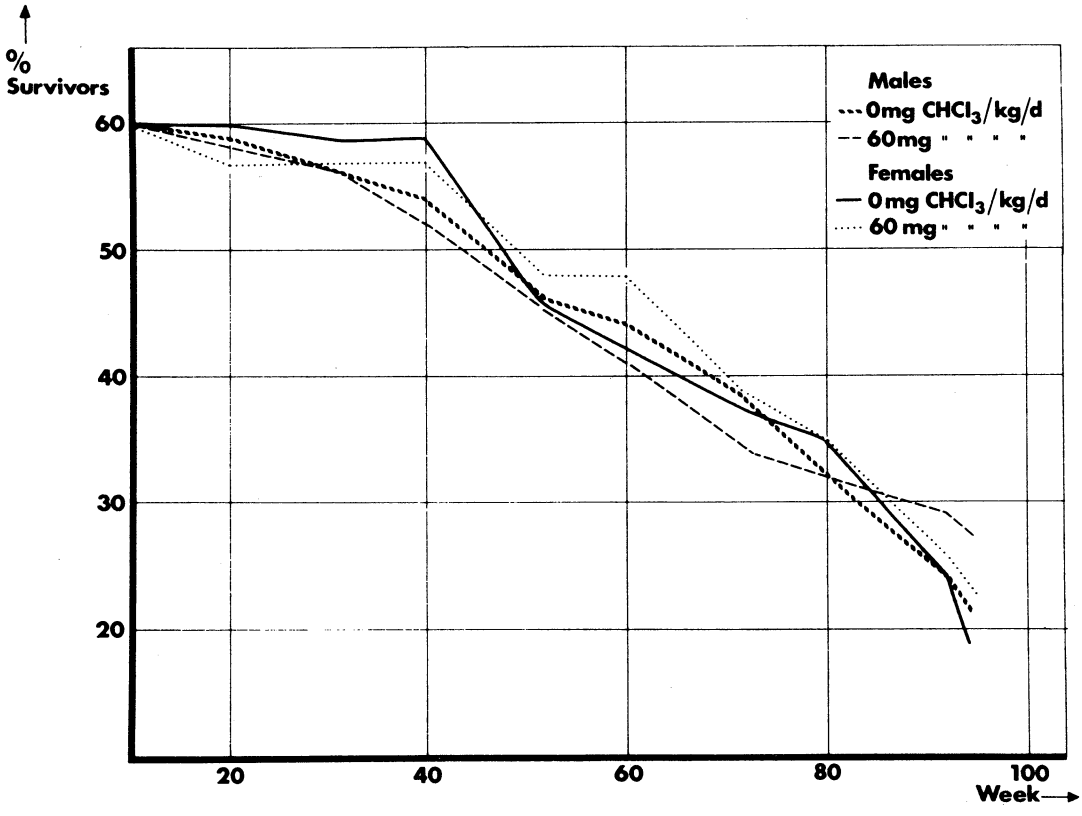

FIGURE 2. Survival of Sprague-Dawley Rats (Expt. II)

severe or severe glomerulo-nephritis were of uncertain significance. No macroscopic or microscopic treatment-related changes were seen in the brains of rats.

Table 4 lists the neoplasms seen. Altogether, 38 chloroform-treated rats and 34 controls had tumours of one or more sites; of these, nine of the treated rats had histologically malignant tumours compared with eight of the controls. Twenty-one females in the treated group developed mammary tumours compared with 16 in the control group; the two groups did not differ with respect to the ages at which animals died with

mammary tumours. Six of the treated female rats were considered to have mammary tumours of histologically malignant type, compared with one control female; the difference was not statistically significant at the 5 percent level (Fisher's Exact Test; $p = 0.094$).

DISCUSSION

The principal findings in the studies reported here were that long-term treatment of Sprague-Dawley rats with toothpaste containing chloro-

TABLE 3. Relative Organ Weight (Group Means as % of Body-wt) in Experiment II

	Males		Females	
Toothpaste: mg $CHCl_3$/kg/d	0	60	0	60
Lungs	0.56	0.95	0.78	0.71
Liver	3.78	3.99	5.01	4.18[a]
Spleen	0.18	0.19	0.25	0.24
Kidneys	0.94	0.99	0.91	0.90
Adrenals	0.015	0.018	0.026	0.022

[a]Significant difference from control $p < 0.01$

form (equivalent to 60 mg $CHCl_3$/kg/d) had no adverse effect on survival and did not significantly influence the age of onset, malignancy or location of tumours. There was no difference between chloroform-treated and control rats in terms of the overall proportion of each group with tumours and no hepatocellular or renal epithelial tumours were seen. The NCI report (1976) shows that, in their study using Osborne-Mendel rats, 54 percent of vehicle control animals had tumours compared with 49 percent of rats in the low-dose groups and 46 percent in the high-dose groups. They saw hardly any liver tumours but drew attention to an apparently dose-related development of renal epithelial tumours in the males.

The rats in the NCI study were housed in the same room as rats being treated with other materials, some of which were volatile compounds known to have carcinogenic activity or to influence the levels of hepatic processing enzymes. Moreover, chloroform was given as a solution in corn oil, so that the findings are of doubtful relevance when considering the long-term effects of chloroform in a water-miscible toothpaste formulation. The NCI study included only 20 rats of each sex in the vehicle control group initially, compared with 50 of each sex at

TABLE 4. Neoplasms recorded in Experiment II

Group	Vehicle Control		60 mg $CHCl_3$/kg/d in toothpaste		Vehicle Control		60 mg $CHCl_3$/kg/d in toothpaste	
No. of rats in group initially	50		50		50		50	
Sex	Male				Female			
	Dying during Experiment	Survivors killed in 95th week	Dying during Experiment	Survivors killed in 95th week	Dying during Experiment	Survivors killed in 95th week	Dying during Experiment	Survivors killed in 95th week
No. examined histologically	37	11	34	15	43	7	36	13
Neoplasms recorded:								
Mammary tumour	—	—	1	—	13	3	14	7
Auditory duct tumour	1	—	1	—	—	—	1	—
Keratoacanthoma of skin	1	1	—	—	—	—	—	—
Sebaceous adenoma	—	1	—	—	—	—	—	—
Prostate adenoma	—	—	—	1	—	—	—	—
Papilloma of skin (in region of preputial gland)	—	1	—	—	—	—	—	—
Subcutaneous fibroma	—	—	—	—	—	—	—	1
Subcutaneous sarcoma (or fibromyxosarcoma)	3	—	—	1	—	—	—	—

TABLE 4. Continued). Neoplasms recorded in Experiment II

Group	Vehicle Control		60 mg $CHCl_3$/kg/d in toothpaste		Vehicle Control		60 mg $CHCl_3$/kg/d in toothpaste	
No. of rats in group initially	50		50		50		50	
Sex	Male				Female			
	Dying during Experiment	Survivors killed in 95th week	Dying during Experiment	Survivors killed in 95th week	Dying during Experiment	Survivors killed in 95th week	Dying during Experiment	Survivors killed in 95th week
No. examined histologically	37	11	34	15	43	7	36	13
Neoplasms recorded (continued):								
Malignant lymphoma	—	—	1	—	—	—	—	—
Lung adenoma	—	—	—	—	—	—	1	—
Adrenal cortex tumour	—	1	1	—	—	—	—	1
Adrenal medulla tumour	1	—	—	—	—	1	1	—
Pituitary chromophobe adenoma	—	—	—	3	3	4	5	4
Seminoma	—	—	—	1	—	—	—	—
Throid adenoma or adenocarcinoma	—	2	—	1	—	1	—	3
Liver cystadenoma	—	—	—	—	—	—	1	—

TABLE 4. (Continued). Neoplasms recorded in Experiment II

Group	Vehicle Control		60 mg $CHCl_3$/kg/d in toothpaste		Vehicle Control		60 mg $CHCl_3$/kg/d in toothpaste	
No. of rats in group initially	50		50		50		50	
Sex	Male				Female			
	Dying during Experiment	Survivors killed in 95th week	Dying during Experiment	Survivors killed in 95th week	Dying during Experiment	Survivors killed in 95th week	Dying during Experiment	Survivors killed in 95th week
No. examined histologically	37	11	34	15	43	7	36	13
Neoplasms recorded (continued):								
Uterine Polyp	—	—	—	—	—	—	—	2
Uterine sarcoma	—	—	—	—	1	—	—	—
Ependymoma (Brain)	1	—	—	—	—	—	—	—
Astrocytoma (Brain)	—	—	—	—	—	—	1	—

TABLE 4. (Continued). Neoplasms recorded in Experiment II

Group	Vehicle Control		60 mg $CHCl_3$/kg/d in toothpaste		Vehicle Control		60 mg $CHCl_3$/kg/d in toothpaste	
No. of rats in group initially	50		50		50		50	
Sex	Male				Female			
No. examined histologically	Dying during Experiment	Survivors killed in 95th week	Dying during Experiment	Survivors killed in 95th week	Dying during Experiment	Survivors killed in 95th week	Dying during Experiment	Survivors killed in 95th week
	37	11	34	15	43	7	36	13
Total rats with tumour at any site.	12		9		22		29	
% rats with tumour at any site.	23		18		44		59	
Total rats with malignant tumour at any site.	6		3		2		6	
% rats with malignant tumour at any site.	13		6		4		12	

each dose level; at the end of the study there were 24 low-dose and 14 high-dose males still alive but only seven vehicle-control males. Since nine out of the 13 renal tumours seen were in chloroform-treated males killed after 111 wk, the paucity of control males concurrently available for observation may have been an important factor. Fears *et al.* (1977) reported a 2.3 percent incidence of kidney tumours in colony control Osborne-Mendel male rats and so the effect of chloroform treatment may have represented an enhancement of the strain-related spontaneous incidence of these tumours.

The dose-related inhibition of hepatic processing enzymes, observed by Puri *et al.* (1971) in rats exposed to chloroform, may result in abnormal exposure of the kidney to naturally-occurring carcinogens of hormonal or dietary origin under experimental conditions of high-dose-level chloroform administration; we have previously drawn attention to this possibility with respect to the kidney tumours seen in ICI male mice (Roe *et al.*, 1978). Certainly there is, at present, no evidence that long-term chloroform treatment produces tumours in any organ of any rodent strain where spontaneous tumours do not occur. If this assessment of the importance of processing enzyme activity is correct, there should be no comparable risk involved in normal human exposure to chloroform as a toothpaste ingredient, since the ingested dose-levels would not suffice to have any significant effect on liver function.

The only change of possible toxicological significance observed in our rat studies was the depression of plasma cholinesterase in female rats. No such effect could be shown against acetyl-β-methylcholine (the definitive substrate for true cholinesterase) but only against butyrylcholine. We have found (unpublished data) that both human and canine plasma exhibit butyrylcholinesterase activity although the repeated administration of toothpaste containing chloroform to dogs did not depress this activity. Thus the effect in female rats on plasma pseudocholinesterase activity appears to be relatively unimportant; it was shown to be reversible after cessation of treatment.

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JEPT MS 419B
Received June 20, 1978
Accepted September 6, 1978