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## SAFETY EVALUATION OF TOOTHPASTE CONTAINING CHLOROFORM I. LONG-TERM STUDIES IN MICE

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*In three experiments, chloroform was administered to mice by gavage in a toothpaste base or in arachis oil, in doses up to 60 mg/kg/d on 6 days/wk for 80 wks. Control groups were left untreated or given vehicle only. In general, there were more survivors in chloroform-treated groups than in the controls. In the case of the males of three strains (C57BL, CBA and CF/1), treatment was associated with no adverse affect on the incidence of any type of neoplasm or any other parameter. In the males but not the females of a fourth strain (IC1) and in doses of 60 mg/kg/d but not of 17 mg/kg/d, exposure to chloroform in toothpaste base as a vehicle was associated with increased incidence of epithelial tumours of the kidney. A more pronounced effect of the same kind was seen in mice given 60 mg CHCl<sub>3</sub>/kg/d in an arachis oil vehicle. This treatment was also associated with a higher incidence and severity of non-neoplastic renal disease. The mechanisms underlying the peculiar strain- and sex-specific susceptibility of IC1 male mice to develop renal tumours when exposed to chloroform remain obscure; spontaneous renal tumours were also seen in vehicle control mice and possible ways in which this tendency may be enhanced by chloroform treatment are discussed. At the dose levels tested, namely 113 and 400 times average human exposure levels from the use of toothpaste (with 3.5 percent chloroform content), no adverse affect was seen in the liver and there was no increased incidence of liver tumours even in the higher liver tumour susceptible CBA strain. At the 17 mg CHCl<sub>3</sub>/kg/d level, equivalent to 113 times average human exposure from toothpaste use, no excess of renal tumours was seen even in males of the peculiarly susceptible IC1 strain.*

### INTRODUCTION

More than a century of human exposure to chloroform as an anaesthetic, solvent, preservative and flavour additive has not revealed any evidence of carcinogenic hazard to man. However, since no deliberate epi-

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9256

demioleological study has been undertaken, a weak association between exposure to chloroform and cancer risk might have been overlooked. For this reason, the laboratory animal studies described in this and two subsequent papers were undertaken.

Prior to communication of the results of the long-term rodent studies on chloroform at the Hazleton Laboratories Inc. (Powers and Voelker, 1976; Renne *et al.*, 1976; National Cancer Institute, 1976) the only adequately controlled study reported was that by Eschenbrenner and Miller (1945). They observed hepatomas in female Strain A mice after repeated oral administration of chloroform in olive oil solution, but only when the doses given were large enough (e. g. 592 mg  $\text{CHCl}_3/\text{kg}$ ) to produce liver necrosis.

The purpose of the studies reported now was to see if exposure to chloroform at dose levels more than 100 times the typical level of ingestion by humans using toothpaste containing 3.5 percent chloroform, but less than those required to produce severe acute liver damage, predisposed to cancer of the liver or any other site.

## MATERIALS AND METHODS

### Selection of Dose Levels

Toothpaste containing 3.5 percent chloroform was first marketed in the UK in 1923. The amounts of chloroform ingested from toothpaste are not easily determined because ingestion is unintentional and variable. A typical quantity of toothpaste used per brushing is 1 g which, in the case of a product containing chloroform, will include 35 mg  $\text{CHCl}_3$ . Atkins (1971) showed that 55 percent of chloroform taken into the mouth as a constituent of toothpaste is immediately exhaled unchanged. This means that if the slurry of toothpaste and saliva in the mouth after toothbrushing was swallowed in its entirety, typical ingestion would be 19.25 mg  $\text{CHCl}_3$ .

Glass *et al.* (1975), using sodium monofluorophosphate as a non-volatile marker, found that small children swallow, on average, 10 to 12 percent of the toothpaste each time they brush their teeth. Adults tend to rinse the mouth more effectively than children and swallow proportionately less of the toothpaste they use (Barnhart *et al.*, 1974); mean adult ingestion is in the region of 3-4 percent of the toothpaste used.

For the present studies, safety margins were calculated with reference to an ingestion level of 12 percent, being the proportion swallowed which would include the upper 90th percentile of adult toothpaste users; the rate of use taken was 1 g twice daily. For a woman of 55kg [the standard mean weight recommended by the Food and Agricultural Organization (F.A.O., 1957)], this is equivalent to an ingested dose level of 0.15 mg  $\text{CHCl}_3/\text{kg}/\text{d}$  (without allowing for loss by volatilization). A 100-fold safety margin therefore requires a no-adverse-effect level of 15 mg/kg/d.

In a preliminary range-finding study, male and female Schofield mice (an outbred Swiss strain) were given toothpaste containing chloroform on 6 d/wk by intragastric intubation at dose-levels equivalent to 0, 60, 150, and 425 mg  $\text{CHCl}_3/\text{kg}/\text{d}$  for 6 wks. All the mice died in the first week of dosing

at 425 mg/kg/d, as did 8/10 males at 150 mg/kg/d; marked retardation of weight-gain of the females at the intermediate dose level was also noted. There were no deaths at 60 mg/kg/d, but moderate retardation of weight-gain in both sexes was indicative of toxicity at this level of exposure. This dose level, representing 400 times the average human exposure level from the twice daily use of toothpaste containing 3.5 percent chloroform, was selected as the upper dose level for the first long-term mouse study and as the appropriate treatment to be given in the subsequent studies.

### Animal Management and Treatment

Toothpaste for administration in these studies complied with the formulation:

	Percentage w/w
Chloroform	3.51
Peppermint Oil	0.25
Eucalyptol	0.50
Glycerol	39.35
Carrageen Gum	0.45
Precipitated Calcium Carbonate	48.53
Sodium Lauryl Sulphate	1.16
Sodium Saccharin	0.03
White Mineral Oil	1.10
Water	5.12
	<b>100.00</b>

Slight modifications were made necessary in some studies by the omission of chloroform or essential oils for various test groups and also to yield a viscosity suitable for intubation.

Chloroform of British Pharmacopoeia quality was used and was therefore not contaminated with other haloalkanes or phosgene. Fresh batches of toothpaste were provided monthly. The toothpaste (assaying 3.33-3.67 percent  $\text{CHCl}_3$ ) had a specific gravity such that a  $\text{CHCl}_3$  dose level of 60 mg/kg was achieved by giving 1 ml of toothpaste/kg. For the dose level of 17 mg  $\text{CHCl}_3$ /kg, toothpaste to the above formula but containing only 1.0 percent  $\text{CHCl}_3$  was given in the same dose volume.

In the first long-term study, random bred male and female albino mice of the ICI strain (ex Smith, Kline & French Ltd.) were quarantined and

allowed to acclimatize for 1 wk, weighed and allocated at random four to a cage to the different groups or to a reserve group. All mice had free access to tap water and to Spratts Laboratory Animals Diet (Autoclaved). Autoclaved softwood sawdust was provided as cage litter. Individual mice were identified by earmark. Animal room temperature and relative humidity were controlled at  $21 \pm 2^\circ$  and  $50 \pm 5$  percent respectively.

Toothpaste, 1 ml/kg body-wt., was given orally by gavage using a blunted metal catheter. Treatment was continued 6 days/wk, at the same time each day, for 80 wk followed by an observation period of 16 wk in the first study. Animals dying during the first 15 wk from natural causes or intubation error were replaced by animals of equivalent weight from the reserve group.

The second and third studies resembled the first procedurally but, in the second study, specific pathogen-free CFLP mice (ICI re-defined) were obtained from Carworth Europe. The third study included:

SPF Mouse strain	Source
ICI	Imperial Chemical Industries Ltd.
CBA	Laboratory Animal Centre, Carshalton
C57BL	Laboratory Animal Centre, Carshalton
CF/1	Carworth Farms, U.S.A.

Treatment commenced in each study when the mice were not more than 10 wks old and continued for 80 wks. The observation period following cessation of treatment varied between 16 and 24 wk according to the number of survivors.

Although random-bred Swiss albino mice of the ICI strain were required for all three studies, sufficient numbers could not be obtained from the same supplier at the time each particular study began. However, all of these mice shared a common origin in the ICI colony at Alderley Park and are described for simplicity as being of the ICI strain in the later sections of this report.

### Observations

Examination for debility or death was carried out twice daily. Any mouse found *in extremis* was immediately killed. Mice found dead or killed *in extremis* were subjected to detailed macroscopic examination and where autolysis was not too advanced, a full range of tissues was preserved in 4 percent formol-saline.

In the first study, the mice were weighed on a cage basis initially at weekly intervals. In the later studies, mice were weighed individually at

weekly intervals for the first 6 mon, then fortnightly for the next 6 mon and thereafter at monthly intervals.

Food consumption was not recorded in the first study but was recorded on a cage basis for the whole of the second study and from weeks 9-12 only in the third study.

The following organ weights were determined after the mice were killed by ether euthanasia: adrenals, kidneys, liver, lungs, and spleen. Ratio of organ-weight to bodyweight was calculated.

All macroscopically-observed tumours and abnormal growths were processed for histopathological examination along with a wide range of available tissues in the first study and brain, lungs, liver and kidney in the

TABLE 1. Design of Experiment I

Group No.	CHCl <sub>3</sub> dose-levels (mg/kg/d)	No. of Mice	
		♂	♀
Vehicle Control	0	104	104
Low Dose	17	52	52
High Dose	60	52	52

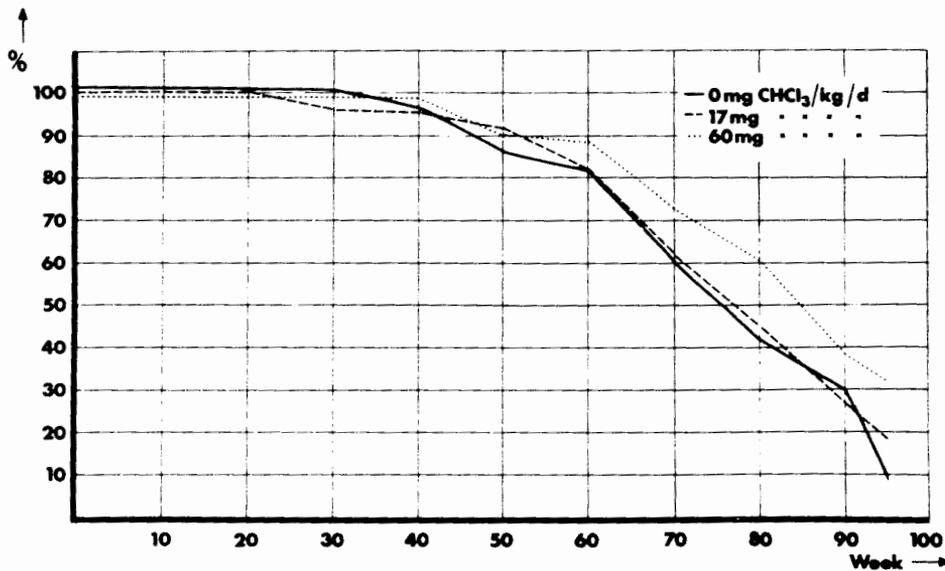


FIGURE 1. Survival of Male ICI Mice (Expt. I)

second and third studies. In all studies, samples representing a wide range of tissues were preserved in 4 percent formol-saline.

In the first study only, a range of haematological estimations was conducted during weeks 21 and 56.

TABLE 2. Design of Experiment II

Group No.	Treatment	Dose-level	No. of ICI Mice	
			♂	♀
1	Untreated	—	52	52
2	Vehicle Control (toothpaste without CHCl <sub>3</sub> peppermint oil and eucalyptol)	1 ml/kg/d	260	—
3	As Group 2 with chloroform	60 mg CHCl <sub>3</sub> / kg/d	52	—
4	As Group 2 with eucalyptol	8 mg eucalyptol/ kg/d	52	—
5	As Group 2 with eucalyptol	32 mg eucalyptol/ kg/d	52	—
6	As Group 2 with peppermint oil	4 mg peppermint oil/kg/d	52	—
7	As Group 2 with peppermint oil	16 mg peppermint oil/kg/d	52	—
8	Alternative non-CHCl <sub>3</sub> toothpaste	1 ml/kg/d	52	52

### Experimental Design

**Experiment I.** The purpose of the first experiment was to study the effects of toothpaste containing 3.5 percent chloroform given to ICI mice at doses equivalent in chloroform content to 113 and 400 times those received by humans brushing their teeth twice daily. The experimental design in this study is shown in Table 1. The vehicle controls in this study received 1 ml/kg/d by oral gavage of unflavoured toothpaste i.e. the formula stated previously minus chloroform, peppermint oil and eucalyptol.

**Experiment II.** The purpose of this experiment was to confirm the findings of Experiment I and to determine the influence of peppermint oil, eucalyptol and chloroform separately. The design is shown in Table 2.

**Experiment III.** The purpose of this experiment was to compare the effects of toothpaste containing 3.5 percent chloroform on male mice of four strains at 400 times human use levels. The design is shown in Table 3.

TABLE 3. Design of Experiment III

Group No.	Mouse Strain	CHCl <sub>3</sub> dose-level <sup>a</sup> mg/kg/d	No. of Mice (all ♂)
1	C57BL	0	52
2	C57BL	60	52
3	CBA	0	52
4	CBA	60	52
5	CF/1	0	52
6	CF/1	60	52
7	ICI	Untreated	100
8	ICI	0	52
9	ICI	60	52
10	ICI	0 <sup>b</sup>	52
11	ICI	60 <sup>c</sup>	52

<sup>a</sup>Groups 1, 3, 5 and 8 received 1 ml/kg/d of toothpaste with peppermint oil and eucalyptol but without chloroform.

<sup>b</sup>Group 10 received 1 ml/kg/d of arachis oil (ex Medina Oil Refineries, London).

<sup>c</sup>Group 11 received 1 ml/kg/d of arachis oil containing 4% v/v CHCl<sub>3</sub>.

## RESULTS

### Experiment I

As shown in Figs. 1 and 2, survival was better in the group given 60 mg CHCl<sub>3</sub>/kg/d than in the other groups, especially in the males. Group mean body-weight in both sexes increased steadily up to week 60 (Figs. 3 and 4), subsequent decline was influenced by the increasing number of deaths but no significant treatment differences were found. The only significant ( $p < 0.05$ ) difference revealed in haematological parameters was in packed cell

volume at week 21 (males given 60 mg  $\text{CHCl}_3/\text{kg}/\text{d}$  = 56 percent, controls = 61 percent).

Absolute and relative organ weights showed no marked differences in group mean values, except for an elevation in liver weight for the low  $\text{CHCl}_3$  dose-level male group (due to one mouse with hepatoma) and in gonad weight for the female control group (due to one mouse with cystic ovaries).

Gross pathology showed that intercurrent respiratory disease was the commonest single cause of death. There were no obvious differences between

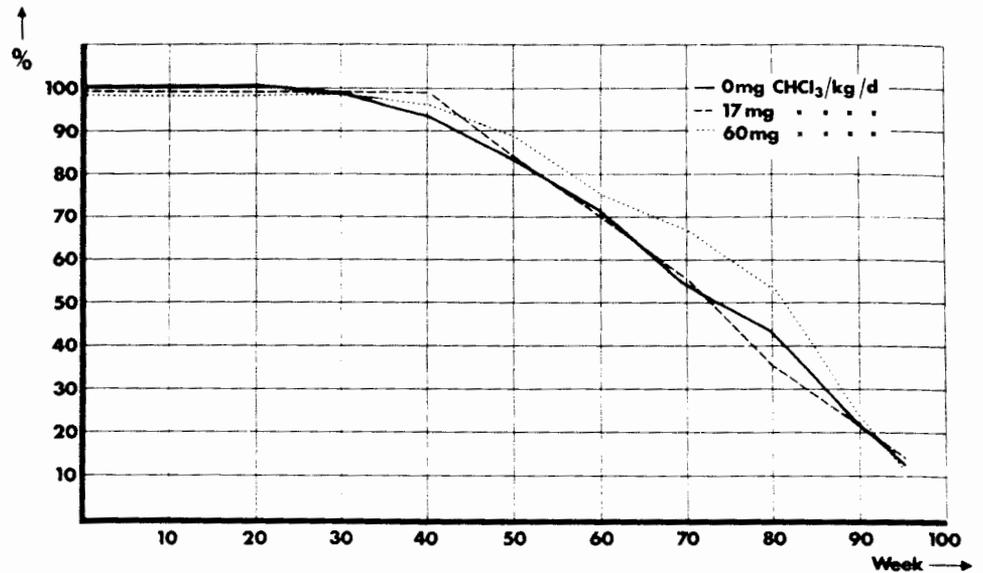


FIGURE 2. Survival of Female ICI Mice (Expt. I)

groups in autopsy findings for mice dying in the course of the study or in mice killed at 96 wk, except that 8 of the males given 60 mg  $\text{CHCl}_3/\text{kg}/\text{d}$  had kidney tumours. Table 4 shows that the overall proportion of mice with tumour at any site was greater in males of the  $\text{CHCl}_3$ -treated groups than in the corresponding controls, but not in treated females compared with control females; the same pattern emerged for mice with malignant tumour at any site. As survival was better in the high-dose  $\text{CHCl}_3$ -treated groups than in the control and low dose groups, no attempt has been made to correct the figures for tumour incidence to take account of survival differences between groups (Peto, 1974). The effect of doing this would have been to increase the incidence in the controls or lower it in the treated groups.

Moderate or severe fatty degeneration of the liver was slightly more prevalent among the chloroform-treated groups than in the controls (Table

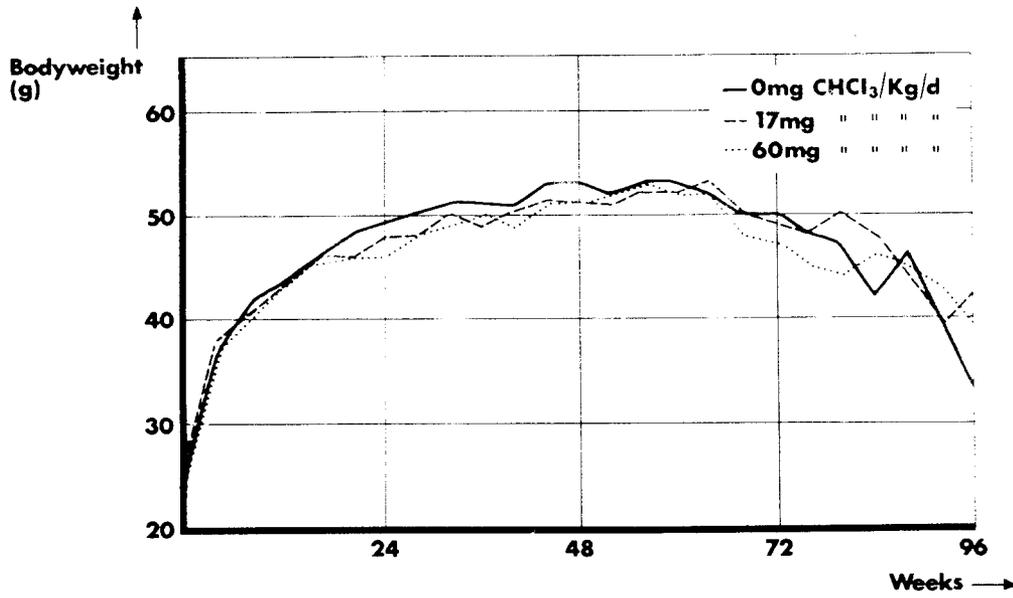


FIGURE 3. Body Weight of Male ICI Mice (Expt. I)

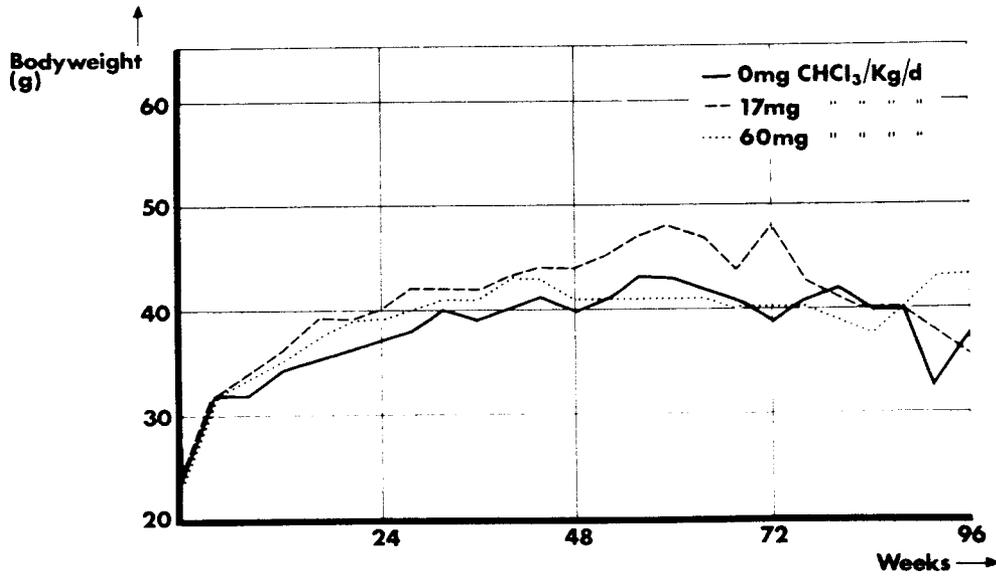


FIGURE 4. Body Weight of Female ICI Mice (Expt. I)

5); the proportion of hepatomas, however, was not significantly increased in these groups. No metastasis or invasion of surrounding structures by any of these tumours was seen. One lung tumour in a male of the high dose group had metastasised widely throughout the lungs but not to other tissues.

Three of the renal tumours in the high-dose group were regarded as possibly malignant and reported as hypernephromas; the remainder were benign cortical adenomas. None of the hypernephromas had invaded other

**TABLE 4.** Incidence of all tumours in Experiment I

CHCl <sub>3</sub> dose-level	0 mg/kg/d		17 mg/kg/d		60 mg/kg/d	
	♂	♀	♂	♀	♂	♀
No. of mice in group	104	104	52	52	52	52
No. examined histologically	72	59	37	35	38	38
Mice with:						
Liver tumours	5	1	6	0	5	0
Lung tumours	7	5	7	2	4	6
Kidney tumours	0	0	0	0	8 <sup>a</sup>	0
Malignant lymphoma (definite)	5	14	7	4	1	8
Malignant lymphoma (probable)	1	0	0	1	2	0
Mammary tumours	0	4	0	0	0	2
Testis tumours	0	0	0	0	2	0
Forestomach tumours	0	0	0	0	1	0
Other neoplasms	4	7	2	4	2	1
Total mice with tumour (any site)	20	29	20	10	21	15
Total mice with malignant tumours (any site)	8	22	9	7	8 <sup>b</sup>	9

<sup>a</sup>Three classed as hypernephroma and the remainder as adenoma.

<sup>b</sup>Locally-invasive lung tumour not included in total for malignant tumours.

tissues. Renal tumours were first encountered in individual mice dying during wk 88 (adenoma) and 92 (hypernephroma); the other six were discovered in apparently healthy males killed at the termination of the experiment at 96 wk. In two of the affected males multiple renal tumours were found. Non-neoplastic renal disease was present in mice of both sexes in all groups but no differences attributable to treatment were discernible.

### Experiment II

Fig. 5 shows that survival was better in the chloroform-treated group than in the vehicle control group. Statistical analysis (one-tailed test) indicated that this difference was significant both for the experimental period as a whole ( $X^2$ , 2.78 > 2.71 for  $X^2$  at  $p < 0.05$ ) and for the period 60-104 wk. ( $X^2$ , 2.87 > 2.71 for  $X^2$  at  $p < 0.05$ ). The overall proportion of deaths in the chloroform-treated group (63 percent) by wk 104 was the lowest for all groups. However, causes of death were essentially similar for all groups.

TABLE 5. Liver Changes in Experiment I

CHCl <sub>3</sub> mg/kg/d	Sex	No. of Livers examined	No. with severe fatty degeneration	No. with severe non- fatty degeneration	No. with liver tumour <sup>a</sup>
0	♂	70	2	6	5
	♀	58	0	3	1
17	♂	35	0	1	6
	♀	34	2	2	0
60	♂	38	3	2	5
	♀	37	2	1	0

<sup>a</sup>None classed as being malignant type.

Body-weight changes in the various groups were similar to those in the untreated controls except for some retardation of weight gain during the early stages of experiment in the groups treated with 60 mg CHCl<sub>3</sub>/kg/d and with 16 mg peppermint oil/kg/d. Food consumption was marginally lower in the CHCl<sub>3</sub>-treated group throughout, and no differences in behaviour or incidence of clinical signs of disease were seen between the groups.

Statistically significant differences from control values were recorded in nearly all groups for various absolute or relative organ weights but the only distinctly treatment-related changes were the lower mean absolute liver and kidney weights in the CHCl<sub>3</sub>-treated mice.

Macroscopic findings at necropsy revealed no clear association between any particular lesion and treatment. Microscopically, interstitial pneumonitis, fatty and non-fatty degeneration of the liver, chronic glomerular nephritis and renal calcification were commonly found, especially in mice dying late in the study or killed at the end of the study. There was no

obvious relationship between the incidence of any of these changes and the treatment given.

Between 61 and 82 percent of mice in each group had one or more neoplasms (see Table 6). The lowest incidence was in the  $\text{CHCl}_3$ -treated group (61 percent) and the next lowest in untreated males (65 percent). Seventy one percent of the vehicle controls and 76 percent of males given an alternative non- $\text{CHCl}_3$  toothpaste (group No. 8) had tumours. The proportion of mice given peppermint oil or eucalyptol which developed tumours was similar to the proportion of vehicle controls that had tumours. The incidence of malignant tumours in male mice was: untreated controls—23 percent; vehicle controls—31 percent;  $\text{CHCl}_3$ -treated group—31 percent; alternative non- $\text{CHCl}_3$  toothpaste group 41 percent. As in the case of

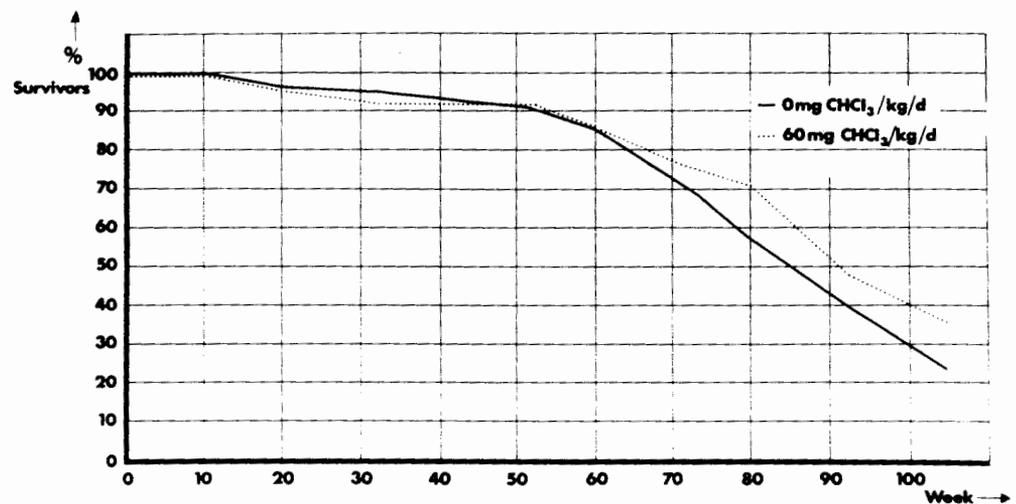


FIGURE 5. Survival of Male ICI Mice in  $\text{CHCl}_3$ -treated and Vehicle Control Group (Expt. II)

Experiment I, the figures for tumour incidence have not been corrected for survival differences, since the  $\text{CHCl}_3$ -treated group again survived better than the controls.

Liver tumours (which occurred in only three of the females) were relatively common in males of all groups. The lowest incidence among males was in the 8 mg eucalyptol/kg/d and 60 mg  $\text{CHCl}_3$ /kg/d groups. Most of the liver tumours in all groups were benign.

The incidence of kidney tumours is shown in detail in Table 7. Although kidney tumours were most common in the  $\text{CHCl}_3$ -treated group, they were also encountered in five other groups. Two of the kidney tumours in the  $\text{CHCl}_3$ -treated mice were regarded as malignant and identified as hypernephromas; all the other kidney tumours were classified as benign

TABLE 6. Incidence of all tumours in Experiment II

	Nil		1 ml tooth- paste base/ kg/d	60 mg CHCl <sub>3</sub> /kg/d	8 mg Eucal- yptol/kg/d	32 mg Eucal- yptol/kg/d	4 mg Peppt. oil/kg/d	16 mg Peppt. oil/kg/d	1 ml alternative non-CHCl <sub>3</sub> toothpaste/kg/d	
	♂	♀	♂	♂	♂	♂	♂	♂	♂	♀
No. of mice in group	52	52	260	52	52	52	52	52	52	52
Mice with:										
Liver tumour	10	3	69	8	5	12	13	14	14	0
Lung tumour	22	24	102	21	30	18	19	20	19	15
Kidney tumour	1	0	6	9	1	0	1	0	2	0
Malignant lymphoma	5	14	37	7	7	5	13	11	12	14
Other neoplasm	4	17	35	3	6	8	5	5	10	9
Total mice with tumour any site	31	40	170	30	56	35	37	34	39	35
No. of mice examined histologically	48	49	240	49	52	47	51	49	51	50

TABLE 6. (Continued). Incidence of all tumours in Experiment II

	Nil		1 ml tooth- paste base/ kg/d	60 mg CHCl <sub>3</sub> /kg/d	8 mg Eucal- yptol/kg/d	32 mg Eucal- yptol/kg/d	4 mg Peppt. oil/kg/d	16 mg Peppt. oil/kg/d	1 ml alternative non-CHCl <sub>3</sub> toothpaste/kg/d	
	♂	♀	♂	♂	♂	♂	♂	♂	♂	♀
No. of mice in group	52	52	260	52	52	52	52	52	52	52
% incidence of mice with tumours at any site	65	82	71	61	69	74	73	69	76	70
No. of mice with malignant tumour <sup>a</sup> (any site)	11	26	75	15	18	11	20	17	21	28
No. of mice with locally invasive lung tumours	8	5	26	5	5	6	3	7	4	3
% of mice with malignant tumour <sup>a</sup> (any site)	23	53	31	31	35	23	39	35	41	56

<sup>a</sup>Classified by histological appearance; not including mice with locally invasive lung tumours.

adenomas, except for a hypernephroma in one mouse given 8 mg eucalyptol/kg/d.

### Experiment III

Marked differences between strains were seen in the number of mice dying in the course of this experiment, the C57BL and CBA males being longer-lived than those of the other two strains. To provide a sufficient proportion of survivors for killing terminally, the surviving CF/1 mice were

TABLE 7. Kidney Tumours in Experiment II

Treatment	Sex	No. of mice for which kidney sections available	No. of mice with kidney tumours	
			Benign	Malignant <sup>a</sup>
Nil	♂	45	1	0
	♀	49	0	0
1 ml Toothpaste base/kg/d	♂	237	6	0
60 mg CHCl <sub>3</sub> /kg/d	♂	49	7	2
8 mg eucalyptol/kg/d	♂	52	0	1
32 mg eucalyptol/kg/d	♂	46	0	0
4 mg peppt. oil/kg/d	♂	50	1	0
16 mg peppt. oil/kg/d	♂	49	0	0
1 ml alternative non-CHCl <sub>3</sub> toothpaste/kg/d	♂	51	2	0
	♀	50	0	0

<sup>a</sup>Classified histologically as hypernephroma.

killed during wk 93 and the ICI survivors in wk 97 and 99. Survivors of the other strains were killed in wk 104. Except in the relatively short-lived CF/1 strain, there were more survivors terminally in the CHCl<sub>3</sub>-treated groups than in the controls (Table 8). There were no apparent differences in causes of death between treated and control animals.

All groups treated with chloroform showed poorer body-weight gain than their respective controls, though the differences were not statistically significant. In this respect, response to chloroform treatment was greater in the ICI and CBA males than in the C57BL and CF/1 males. Food consumption records showed only minor differences between treated and control mice in the various strains. No overt signs of reaction to treatment were seen at any time during the study.

Because of the relatively high incidence of tumours and the wide

TABLE 8. Survivors at Time of Terminal Kill in Experiment III

Strain	Chloroform mg/kg/d	Percent Survivors
C57BL	0	52
C57BL	60	69
CBA	0	69
CBA	60	79
CF/1	0	21
CF/1	60	12
ICI	0	27
ICI	60	31
ICI	Untreated	19
ICI	0 <sup>a</sup>	21
ICI	60 <sup>b</sup>	17

<sup>a</sup>Arachis oil

<sup>b</sup>Dissolved in arachis oil

variety of age-dependent changes, comparison of group mean absolute and relative organ weights gave a confusing picture. Gross pathological findings reflected a diverse range of changes with no obvious differences between treated and control groups. In CF/1 and ICI mice that died early in the experiment, the respiratory and urinary tracts were most often affected. Staphylococcal infection appeared to have been responsible for some early deaths of ICI mice and fighting contributed to the incidence of early death in CF/1 animals.

The incidence of glomerular nephritis was significantly lower ( $p < 0.001$ ,  $X^2$  test) in C57BL mice than in mice of the other three strains.

TABLE 9. Incidence of Neoplasms in Experiment III

Strain (♂ only)	CHCl <sub>3</sub> mg/kg/d (in toothpaste base with pepper- mint oil and eucalyptol)	No. of mice examined histologically	No. of mice with									% mice with tumour at any site	% mice with malignant tumour at any site <sup>c</sup>
			Kidney tumour		Liver tumour		Lung tumour			Malignant Lymphoma	Other neoplasm		
			Benign	Mal.	Benign	Mal.	Benign	Locally invasive	Mal.				
C57BL	0	46	0	0	2	0	1	1	0	10	2	35	26
C57BL	60	51	0	0	0	0	3	1	0	7	1	24	16
CBA	0	51	0	0	35	2	10	3	0	2	1	82	8
CBA	60	51	0	0	29	0	5	3	0	0	1	65	2
CF/1	0	45	0	2	4	0	7	2	0	6	4	36	24
CF/1	60	48	0	1	5	0	9	1	2	6	4	46	25
ICI	0	49	1	0	6	1	4	0	0	4	2	35	10
ICI	60	47	2	3	9	0	6	1	0	5	4	51	21
ICI	Untreated	83	0	0	8	0	6	1	0	11	3	34	14
ICI	0 <sup>a</sup>	50	1	0	7	2	3	1	1	8	6	48	24
ICI	60 <sup>b</sup>	48	3	9	7	1	2	1	0	8	1	42	33

<sup>a</sup>Arachis oil.<sup>b</sup>Dissolved in arachis oil.<sup>c</sup>Classified by histological appearance and/or evidence of metastasis; locally-invasive lung tumors not included in totals for malignant tumours.

Treatment with chloroform was associated with significantly ( $P < 0.001$ ,  $X^2$  test) higher incidence of moderate to severe kidney changes in the CBA and CF/1 males than in the corresponding controls. Neoplasia in addition to respiratory and urinary tract diseases, contributed to the causes of death in the later stages of the study in all four strains (see Table 9). Of the 191 vehicle-control mice of all four strains examined histologically, 32 (16.8 percent) had malignant tumours at one or more sites. In the case of mice treated with 60 mg  $\text{CHCl}_3/\text{kg}/\text{d}$  in toothpaste, 31/197 (15.8 percent) had tumours of malignant type. Treatment with chloroform was not associated with any increase in the incidence of liver and lung tumours encountered in the vehicle-treated controls. Malignant lymphomas were observed in all strains, but the proportion affected was not increased by chloroform treatment in any strain. Twelve of the ICI males exposed to chloroform dissolved in arachis oil developed kidney tumours and in nine of these the tumours were regarded as malignant. By comparison only one of the ICI males exposed to arachis oil had a kidney tumour, and this was benign. Chloroform administration in toothpaste base was less productive of kidney tumours (five animals with tumours of which three had malignant tumours compared with one vehicle-treated control with a benign tumour). The three animals given chloroform in toothpaste base that developed malignant kidney tumours all survived to the end of the experiment. By contrast, five out of the nine mice in the group that received chloroform in arachis oil and developed malignant kidney tumours died before the study was terminated. No kidney tumours were seen in C57BL or CBA mice; in CF/1 males, two vehicle controls and one chloroform-treated mouse were deemed to have malignant kidney neoplasms. Chloroform in arachis oil significantly ( $p < 0.05$ ,  $X^2$  test) increased the incidence of moderate to severe kidney disease compared with arachis oil only controls.

## DISCUSSION

At the time the studies on chloroform reported in this paper began, an increased incidence of liver tumours in mice might have been expected on the basis of the earlier report by Eschenbrenner and Miller (1945). The three studies carried out, however, have not substantiated this in any sense; combining the data from the three studies, the incidence of liver tumours was 72/420 (17 percent) for vehicle controls of the ICI strain and 27/171 (16 percent) for ICI mice given 60 mg  $\text{CHCl}_3/\text{kg}/\text{d}$  in toothpaste. Moreover, in the CBA strain, which was chosen on account of the high spontaneous incidence of liver tumours, chloroform treatment did not enhance this incidence.

These negative findings are consistent with the hypothesis that the increased incidence of liver tumours seen by Eschenbrenner and Miller (1945) depended on their giving doses of chloroform sufficient to cause necrosis of the liver. The findings of Roe *et al.* (1968), who observed no increase in liver tumours in the treated animals in their study using neonatal mice, are similarly consistent with this hypothesis.

Our findings are in marked contrast to those reported by the National

Cancer Institute (1976); large numbers of liver tumours were described in male and female B6C3F<sub>1</sub> mice treated with chloroform dissolved in corn oil. The striking difference in liver-tumour incidence between our studies and the NCI mouse study may be related to the differences in dose-levels given, the different vehicles used and the different criteria applied to the diagnosis of hepatic lesions. A comment in the Federal Register (1976), referring to the B6C3F<sub>1</sub> mice used in the NCI study, indicated that doses as high as 3620 and 5000 mg CHCl<sub>3</sub>/kg body-wt were needed to produce lethality in males and females, respectively. The ability of these mice to withstand doses of chloroform more than twice as great as those previously recorded for the oral LD<sub>50</sub>, recalls the findings reported by Reuber and Glover (1970); these authors showed that rat strains which survived relatively high dose-levels of carbon tetrachloride developed hepatocellular carcinoma whereas other strains which died early showed no evidence of carcinoma development. However, our findings would appear to be consistent with those of Eschenbrenner and Miller (1945), indicating that doses of chloroform below those required to cause severe liver damage, are unlikely to involve any increased risk of liver cancer.

In three separate experiments in mice exposed for long periods to chloroform as a toothpaste ingredient at dose levels corresponding to 400 times the average quantity likely to be ingested during twice daily tooth-brushing, we found no clear evidence that chloroform increased the overall risk of development of neoplasms at any site, except that in the males of one of the four strains studied (the ICI strain) exposure to chloroform was consistently associated with increased incidence of both benign and malignant kidney tumours. Even though ICI mice were obtained from three different suppliers, they all responded similarly with regard to kidney tumour incidence; this seemed to emphasize the strain-specificity of the response, since it did not occur in the other strains tested. Most of the kidney tumours in ICI males were discovered at necropsy in apparently healthy mice killed at the end of a study, except when chloroform was given in an arachis oil vehicle.

A peculiar susceptibility of male mice of certain strains to the nephrotoxic action of chloroform has been reported by several workers (e. g. Deringer *et al*, 1953; Shubik and Ritchie, 1953; Culliford and Hewitt, 1957) and a connection between sex-, strain- and species-specific susceptibility to acute nephrotoxic effects and to the induction of neoplasms of the kidney would not have been surprising. However, non-neoplastic renal disease was not especially common in ICI males compared with ICI females or the males of other strains; nor was it more marked in mice with kidney tumours than in mice without them.

In two of our three long-term experiments, kidney tumours were seen in vehicle control ICI males as well as in the chloroform-treated animals. It is, therefore, possible that these mice are susceptible to renal carcinogens of hormonal or dietary origin and that chloroform treatment enhances the tendency to develop spontaneous kidney tumours. Pound, *et al*, (1973) showed that prior administration of carbon tetrachloride increased the yield of kidney tumours in rats given dimethylnitrosamine, which may have been a consequence of the inhibition of hepatic processing enzymes by carbon

tetrachloride. Cawthorne *et al.* (unpublished) found that male ICI mice were relatively deficient in hepatic processing enzyme activity compared with the males of the other strains used in our studies; they estimated the level of hexobarbitone oxidase activity *in vitro* ( $\mu$  moles/g liver/h) as 1.47 for ICI males, 7.79 for CF/1 males, 11.31 for CBA males and 6.98 for C57BL males. Hepatic processing enzyme activity would be expected to be depressed by chloroform administration (Puri *et al.*, 1971) and it would therefore not be surprising to find, especially in the ICI males, an enhanced risk of developing spontaneous kidney tumours elicited by naturally-occurring carcinogens. No such risk would seem likely at dose levels of chloroform insufficient to have a substantial effect on metabolic activity in the liver, and we did not see any kidney tumours in Experiment I at a dose level of 17 mg  $\text{CHCl}_3/\text{kg}/\text{d}$ .

An alternative explanation is that deficient hepatic metabolism of chloroform itself might lead to unusually high concentrations reaching the kidney, where susceptibility to its nephrotoxic effects would possibly lead to regenerative hyperplasia and eventually to neoplasia. Such a pattern of response in the kidney does not seem to have been reported previously and the possibility could only be explored by means of a study including interim sacrifice of some of the animals. Metabolic studies by Taylor *et al.* (1974) have, however, confirmed that activity is concentrated in the renal cortex of male mice after giving  $^{14}\text{C}$ -labeled chloroform by gavage.

The suggestion that the increased incidence of kidney tumours in chloroform-treated ICI male mice may not have resulted from a mutation-mediated carcinogenic action is compatible with the negative findings reported for bacterial mutagenicity by Uehleke *et al.* (1977) and Simmon *et al.* (1977). The lack of mutagenic action reported by these authors appears to indicate that chloroform has no direct effect on DNA.

Our studies have consistently shown better survival in mice treated with chloroform as a toothpaste ingredient than in the vehicle controls, which led us to suppose that gastro-intestinal antimicrobial activity might have been involved. Comparable benefit in terms of survival was not achieved when chloroform was given dissolved in arachis oil and the mice receiving this treatment had a greater incidence of kidney tumours, especially with respect to malignancies in the animals dying in the course of the experiment. Eschenbrenner and Miller (1945) gave chloroform to mice in the form of a solution in olive oil, a vehicle similar to arachis oil; the NCI study (1976) utilized corn oil as the vehicle. The fact that these investigations did not lead to an excess of kidney tumours in mice reinforces the view that the phenomenon is highly strain specific.

## REFERENCES

- Atkins, P. R.: M. Sc. Thesis The Application of a Halogen Selective Detector to the Measurement of Chloroform in Respired Air during Toothbrushing. Bath University of Technology, 1971.
- Barnhart, W. E., Hiller, L. K., Leonard, G. J. and Michaels, S. E.: Dentifrice Usage and Ingestion among Four Age Groups J. dent. Res. 53: 1317, 1974.

- Cawthorne, M. A., Palmer, E. D., Bunyan, J. and Green, J.: Preliminary Report of Biochemical Studies on Strain Differences in the Occurrence of Renal Neoplasms in Mice Given Chloroform. Unpublished. 1969.
- Culliford, D. and Hewitt, H. B.: The Influence of Sex Hormone Status on the Susceptibility of Mice to Chloroform-Induced Necrosis of the Renal Tubules. *J. Endocrinol.* 14: 381, 1957.
- Deringer, M. K., Dunn, T. B., and Heston, W. E.: Results of Exposure of Strain C3H Mice to Chloroform. *Proc. Soc. Exp. Biol. & Med.* 83: 474, 1953.
- Eschenbrenner, A. B., and Miller, E.: Induction of Hepatomas in Mice by Repeated Oral Administration of Chloroform with Observations on Sex Differences. *Jnl. Nat. Cancer Inst.* 5: 251, 1945.
- Food and Agriculture Organization. Calorie Requirements. Nutritional Studies No. 15 (Rome: FAO), 1957.
- Food and Drug Administration. Chloroform as an Ingredient of Human Drug and Cosmetic Products. *Fed. Reg.* 41: 26842, 1976.
- Glass, R. L., Peterson, J. K., Zuckerberg, D. A. and Naylor, M. N.: Fluoride Ingestion Resulting from the Use of Monofluorophosphate Dentifrice by Children. *Brit. Dent. J.* 138: 423, 1975.
- National Cancer Institute. Report on Carcinogenesis Bioassay of Chloroform March 1, 1976.
- Peto, R.: Guidelines on the Analysis of Tumour Rates and Death Rates in Experimental Animals. *Br. J. Cancer* 29: 101, 1974.
- Pound, A. W., Lawson, T. A., and Horn, L.: Increased Carcinogenic Action of Dimethylnitrosamine after Prior Administration of Carbon Tetrachloride. *Br. J. Cancer* 27: 451, 1973.
- Powers, M. B., and Voelker, R. W.: Evaluation of the Oncogenic Potential of Chloroform by Long-term Oral Administration in Rodents. *Toxicol. Appl. Pharmacol.* 37: 179, 1976.
- Puri, S. K., Fuller, G. C. and Lal, H.: Effect of Chloroform Inhalation on Barbiturate Narcosis and Metabolism in Normal Phenobarbital Pretreated Rats. *Pharmacol. Res. Comm.* 3: 247, 1971.
- Renne, R. A., Firrell, J. F., Voelker, R. E. and Powers, M. B.: Pathology of Long-Term Administration of Chloroform in Rodents. *Toxicol. Appl. Pharmacol.* 37: 179, 1976.
- Reuber, M. D. and Glover, E. L.: Cirrhosis and Carcinoma of the Liver in Male Rats Given Subcutaneous Carbon Tetrachloride. *J. Nat. Cancer Inst.* 44: 419, 1970.
- Roe, F. J. C., Carter, R. L. and Mitchley, B. C. V.: *A. R. Brit. Emp. Cancer Campaign.* 46: 13, 1968.
- Simmon, V. F., Kauhanen, K. and Tardiff, R. G.: Mutagenic Activity of Chemicals Identified in Drinking Water. 2nd Internat. Env. Mut. Soc. Mtg., Edinburgh, 1977.
- Shubik, P. and Ritchie, A. C.: Sensitivity of Male dba Mice to the Toxicity of Chloroform as a Laboratory Hazard. *Science*, 117: 285, 1953.
- Taylor, D. C., Brown, D. M., Keeble, R. and Langley, P. F.: Metabolism of Chloroform II. A Sex Difference in the Metabolism of (<sup>14</sup>C) Chloroform in Mice. *Xenobiot.* 4: 165, 1974.
- Uehleke, H., Greim, H., Kramer, M. and Werner, T.: Metabolic Activation of Haloalkanes and Tests *In Vitro* for Mutagenicity. *Xenobiot.* 7: 393, 1977.

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