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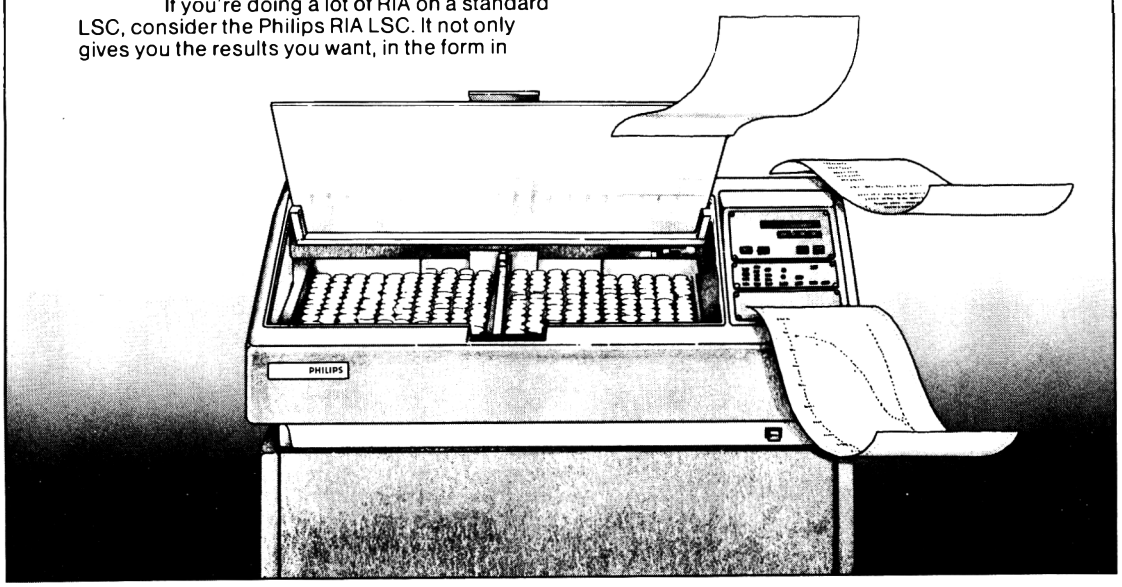

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Research Section

ABSORPTION, DISTRIBUTION AND EXCRETION OF [^{14}C]CARMOISINE IN MICE AFTER ORAL AND INTRAVENOUS ADMINISTRATION

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(Received 10 February 1981)

Abstract—Male Swiss albino mice (CD-1) were given single doses of [^{14}C]carmoisine by stomach tube (200 mg/kg, 6 μCi) or iv injection (200 mg/kg; 0·7 μCi). The plasma and tissue kinetics of the compound were studied by monitoring the decay of radioactivity in the plasma, gastro-intestinal tract, liver, kidney, lung, testes, spleen and gall bladder. The faeces and urine of mice placed in individual metabolism cages were collected between 4 and 96 hr after dosing. After oral administration peak levels of radioactivity occurred in the plasma and the liver, lungs, testes and spleen 8 hr after dosing. Radioactivity was almost completely (98%) excreted in the faeces and urine within 16–32 hr after oral dosing. The plasma [^{14}C]radioactivity decay curve after iv administration indicated a very rapid distribution of the compound into the tissues ($t_{1/2}$ = 10 min) and an efficient excretion, mostly through the gastro-intestinal tract (92%), which was complete 48 hr after dosing.

INTRODUCTION

Carmoisine (Azo Rubine; Ext. D & C Red No. 10; C.I. (1956) No. 14720; E122) is the disodium salt of 2-(4′-sulpho-1′-naphthylazo)-1-naphthol-4-sulphonic acid. It is one of the dyes currently permitted for food use in the EEC member countries, but not in the USA. The available toxicity data on carmoisine show that the azo dye is relatively nontoxic. The acute ip LD_{50} was 0·9 g/kg in mice and 1·0 g/kg in rats (Gaunt, Farmer, Grasso & Gangolli, 1967). The LD_{50} in mice after iv injection was 0·8 g/kg (Hecht, 1966). Acute oral doses of 8 g/kg in the mouse and 10 g/kg in the rat were tolerated without adverse effect (Gaunt *et al.* 1967). No adverse effects on general health, growth, food consumption, haematological parameters or liver and kidney function were found in rats fed carmoisine for 90 days at levels ranging from 0·05 to 1·0% in the diet. An elevated relative kidney weight was found at the 1% dose level in females. The type and incidence of histological changes were comparable in control and test groups and a no-effect level of 0·5% in the diet was established (Gaunt *et al.* 1967).

The no-effect level was at least 1·0 g/kg/day, or 3–4%, in miniature pigs fed carmoisine at dose levels of up to 1000 mg/kg/day for 3 months (Gaunt, Grasso, Kiss & Gangolli, 1969). A similar lack of toxic effects after oral administration was found in a 1-yr feeding study in rats in which carmoisine was fed at 0·35, 0·8 or 2·0% in the diet. The no-effect level was 0·8%; at the 2·0% level there was an increased incidence of several mild subclinical conditions (Holmes, Pritchard & Kirschman, 1978a).

Reproduction studies, including teratogenicity studies, using diets containing 0·35, 0·8 or 2·0% of the azo dye, did not reveal any compound-related adverse effects (Holmes, Pritchard & Kirschman, 1978b). Carmoisine did not show any carcinogenic potential when administered to mice at dietary levels of up to 1·25% in an 80-wk study (Mason, Gaunt, Butterworth, Hardy, Kiss & Grasso, 1974). Neither were any carcinogenic effects observed when mice were given twice-weekly sc injections of 0·1 ml of a 3–6% suspension of carmoisine in water for 360 days with subsequent observation for periods in excess of 630 days (Bonser, Clayson & Jull, 1956). The aim of the present work was to study the absorption, fate and excretion of carmoisine in mice following oral and iv administration of the dye.

EXPERIMENTAL

Materials and animals. [1, 4, 5, 8, 1′, 4′, 5′, 8′,-^{14}C]Carmoisine (Fig. 1), 99% radiochemically pure, with a specific activity of 5 mCi/mmol was purchased from the Radiochemical Centre Ltd, Amersham, Bucks, UK and was generously supplied by Davide Campari S.p.A., Milan. Unlabelled carmoisine, complying fully with the EEC and FDA purity requirements and having a dye content greater than 87%, was obtained from B.V. Nederlanse Kleurstoff-Industrie, Amersfoort, Netherlands, and was used to adjust the dose of [^{14}C]carmoisine to the desired specific activity.

Male Swiss albino mice (CD-1) obtained from Charles River Inc. (Calco, Como) and weighing 22–28 g were used in all of the experiments.

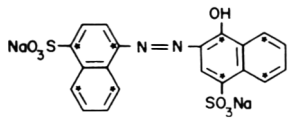

Fig. 1. Chemical structure of [1, 4, 5, 8, 1', 4', 5', 8'-^{14}C]carmoisine.

Experimental design and conduct. In the oral experiment all of the animals were fasted for 12 hr and were then given 200 mg [^{14}C]carmoisine/kg body weight (6·0 μCi) in 0·2 ml of water, by gavage. Groups of mice were decapitated after 5, 10, 15 or 30 min or 1, 2, 4, 8, 16, 32, 64 or 96 hr and blood was collected in heparinized tubes. In another series of experiments mice that had been fasted for 12 hr were each injected in the tail vein with 0·7 μCi [^{14}C]carmoisine in 0·15 ml of water. This corresponded to a dose of 200 mg carmoisine/kg. In both the oral and the iv studies untreated mice were used as controls.

The gall bladder, liver, testes, lungs, spleen, brain, kidneys and gastro-intestinal tract were analysed for radioactivity. Tissue homogenates were also prepared from control animals. Mice from which faeces and urine were collected were housed individually in metabolism cages (Techniplast, Buguggiate, Varese) for 4–96 hr after dosing and were fed 8 hr after dosing; they were given water *ad lib.*

Determination of radioactivity. Tissues, blood, urine and faeces were frozen on solid CO_2 immediately and kept at −20°C until radioactivity measurement which was carried out on duplicate samples. Tissue homogenates (50 μl) prepared in four volumes of water were added to 1·0 ml of Lumasolve (Lumac System A.G., Basle, Switzerland) and digested by shaking at 50°C for 30 min. After cooling on ice, the sample was added to 5 ml of the scintillation solution (Lipoluma; Lumac System A.G.) and radioactivity was determined using a Packard Tri-Carb 3255 liquid scintillation spectrometer using external standard channel ratios to correct for sample quenching.

Aliquots (50 μl) of blood were added to 0·5 ml of Lumasolve–isopropanol (1:2, v/v) solution and shaken for 60 min at 50°C. After dropwise addition of 0·5 ml 35% (v/v) H_2O_2 to bleach the sample the vials were kept for 30 min at room temperature and 10 ml of Lumagel–0·5 N-HCl (9:1, v/v) were added before counting. Urine aliquots (0·1 ml) were counted by liquid scintillation after addition of 10 ml of Lumagel (Lumac System A.G.). Faeces were suspended in four volumes of water and stirred until completely dispersed. To 50 μl of the mixture 0·5 ml of Lumasolve–isopropanol (1:2, v/v) was added and the samples were incubated at 50°C overnight. Cooled samples were bleached with 0·5 ml 35% (v/v) H_2O_2; 15 ml of Lumagel–0·5 N-HCl (9:1) was added before counting.

RESULTS

For 96 hr following the administration of ^{14}C-carmoisine by either oral or iv routes, the animals showed no signs of abnormal appearance or behaviour. Food and water consumptions were similar in treated animals and controls. There were no differences in absolute or relative organ weights between treated and control mice.

The levels of radioactivity in the plasma after oral administration of [^{14}C]carmoisine is shown in Fig. 2. The half-life, calculated from the regression line of the β-phase, was found to be 39 hr. The maximum amount of circulating radioactivity corresponded to about 0·2% of the dose as calculated from the concentration of carmoisine in the plasma and the mean amount of plasma in mice.

The radioactivity recovered in the tissues of mice treated orally with [^{14}C]carmoisine is shown in Tables 1 and 2. The peak of radioactivity was found in the blood and in the liver, lungs, testes and spleen 8 hr after administration of the dye. In the lung, testes and spleen significant levels of radioactivity were not detected until 4 hr after administration (Table 2). At no time was radioactivity detectable in the brain.

Levels of radioactivity present in the blood after iv administration are shown in Fig. 3. The calculated log Cp = f(t) (where Cp is the concentration of the substance in the plasma and t is time) regression lines of the β-phase (elimination phase) and of the residuals of the α-phase (distribution phase), showed a good fit with a two-compartment open model. The half-life of the α-phase was calculated as 10 min whereas a value of 13·9 hr was calculated for the elimination phase. The area under the plasma decay curve was found to

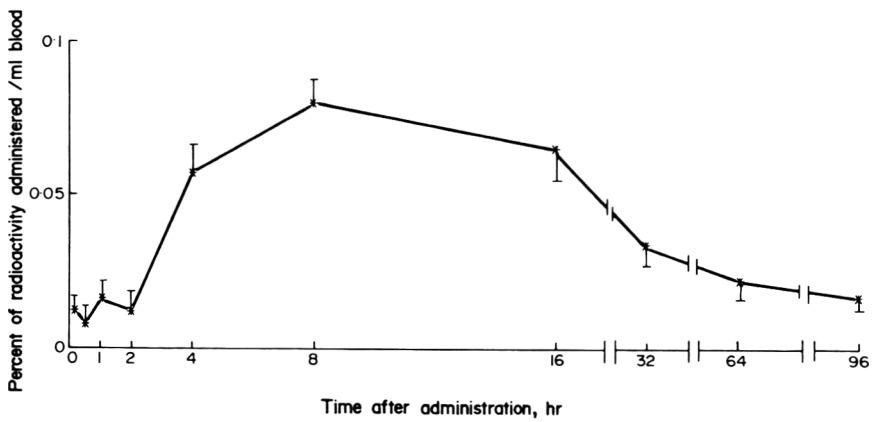

Fig. 2. Disappearance of ^{14}C-activity from the blood of mice following oral administration of a single dose of [^{14}C]carmoisine (200 mg/kg; 6·0 μCi). Each point represents the mean ± SEM for groups of between three and ten mice.

Table 1. *Distribution of radioactivity in the gastro-intestinal tract, liver, kidney and gall bladder of mice following administration of a single oral dose of [^{14}C]carmoisine (200 mg/kg; 6·0 μCi)*

Time after dosing	^{14}C-activity (dpm × 10^4/g) in			
	Gastro-intestinal tract	Liver	Kidney	Gall bladder*
5 min	300 ± 20 (5)	0·33 ± 0·08 (5)	0·17 ± 0·03 (5)	ND† (5)
10 min	300 ± 20 (5)	0·41 ± 0·08 (4)	0·31 ± 0·08 (5)	0·02 ± 0·005 (5)
15 min	330 ± 20 (5)	0·41 ± 0·05 (3)	0·32 ± 0·02 (3)	0·05 ± 0·01 (3)
30 min	250 ± 20 (8)	0·41 ± 0·07 (5)	0·40 ± 0·07 (5)	0·03 ± 0·01 (5)
1 hr	280 ± 30 (10)	0·94 ± 0·08 (7)	0·35 ± 0·16 (7)	0·1 ± 0·03 (5)
2 hr	320 ± 20 (7)	0·32 ± 0·04 (4)	4·6 ± 0·10 (4)	0·2 ± 0·07 (7)
4 hr	320 ± 30 (7)	2·13 ± 0·83 (4)	2·14 ± 0·78 (4)	19·2 ± 10·9 (4)
8 hr	190 ± 30 (8)	2·53 ± 1·02 (6)	3·23 ± 0·14 (6)	11·0 ± 5·8 (4)
16 hr	100 ± 30 (10)	1·12 ± 0·42 (10)	2·01 ± 0·47 (10)	6·8 ± 4·4 (7)
32 hr	20 ± 10 (10)	0·75 ± 0·24 (6)	2·38 ± 0·12 (5)	15·2 ± 7·1 (5)
64 hr	3 ± 2 (5)	0·36 ± 0·23 (5)	0·95 ± 0·55 (5)	16·6 (2)
96 hr	0·1 (2)	0·08 (2)	0·07 (2)	ND (2)

*dpm × 10^4/gall bladder.
†ND = Not detected. Values were not significantly different ($P > 0·05$ by one-way analysis of variance) from values for corresponding tissue homogenates from control animals.
Values are means ± S.E.M. for the number of animals indicated in brackets. Radioactivity measurements for each tissue sample were made in duplicate.

be of the same order as that observed after oral administration (Fig. 2). This result and the high concentration of radioactivity in the gastro-intestinal tract (Table 3) soon after administration seems to indicate a high affinity of the dye for the intestine. Radioactivity was found in all of the tissues that were analysed (Tables 3 & 4), but an efficient decay was observed and no accumulation occurred in any tissue, as happened after oral administration.

Faecal and urinary recoveries of [^{14}C]carmoisine expressed as percentages of the doses administered are shown in Tables 5 and 6. Excretion of carmoisine following oral administration was essentially complete after 32 hr. Similarly, after iv injection of [^{14}C]carmoisine most of the radioactivity (76%) had been excreted 24 hr after dosing, and all of the radioactivity was accounted for after 48 hr.

DISCUSSION

The low levels of radioactivity found in the tissues of mice after oral administration of carmoisine are consistent with the generally accepted view that azo dyes are not readily absorbed from the gastro-intestinal tract because of their hydrophilic nature. In addition, data from both the oral and the iv studies indicate that the rates of disappearance of the radioactivity from the plasma are similar to those from tissues, suggesting that there is no preferential concentration of the dye or of its metabolites in any particular tissue.

Previous studies by other authors have demonstrated an active excretion through the bile of both carmoisine and other azo dyes after iv injection of the compounds into bile-duct cannulated rats (Ryan & Wright, 1961).

In mice, iv injected [^{14}C]carmoisine seems to follow a similar fate. At least part of the radioactivity present in the bile was associated with unchanged carmoisine. This was indicated by the red colour seen in the bile of mice killed at intervals up to 4 hr after dosing. Moreover a saturation process is suggested, because as early as 10 min after administration a level of about 2% was reached in the gall bladder. This level remained constant for at least 2 hr while circulating radioactivity was decreased to 0·1% of the injected dose and radioactivity in the gastro-intestinal tract was increasing. Saturation of the biliary excretion process may be responsible for the long half-life

Table 2. *Distribution of radioactivity in the lung, testes and spleen of mice following administration of a single oral dose of [^{14}C]carmoisine (200 mg/kg; 6·0 μCi)*

Time after dosing (hr)	^{14}C-activity (dpm × 10^4/g) in		
	Lungs	Testes	Spleen
4	0·86 ± 0·15 (4)	0·45 ± 0·19 (4)	0·16 ± 0·03 (4)
8	1·30 ± 0·59 (3)	0·55 ± 0·17 (6)	0·32 ± 0·08 (6)
16	1·00 ± 0·34 (7)	0·22 ± 0·06 (10)	0·23 ± 0·06 (10)
32	ND* (3)	0·27 ± 0·12 (6)	0·21 ± 0·09 (6)
64	ND (3)	0·31 (2)	0·24 ± 0·01 (5)
96	ND (2)	ND (2)	ND (2)

*ND = Not detected. Values were not significantly different ($P > 0·05$ by one-way analysis of variance) from values for corresponding tissue homogenates from control animals.
Values are means ± S.E.M. for the number of animals indicated in brackets. Radioactivity measurements for each tissue sample were made in duplicate.

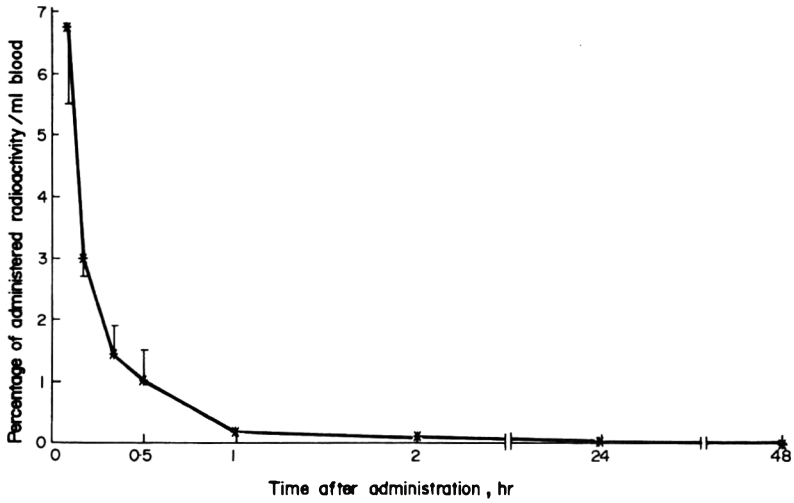

Fig. 3. Disappearance of ^{14}C-activity from the blood of mice given a single iv injection of [^{14}C]carmoisine (200 mg/kg; 0·7 μCi). Each point is the mean ± SEM for groups of at least three animals.

Table 3. *Distribution of radioactivity in the gastro-intestinal tract, liver, kidney and gall bladder of mice after iv injection of a single dose of [^{14}C]carmoisine (200 mg/kg; 0·7 μCi)*

Time after dosing	^{14}C-activity (dpm × 10^4/g) in the			
	Gastro-intestinal tract	Liver	Kidney	Gall bladder*
5 min	5·5 ± 0·8	16·2 ± 3·2	2·7 ± 0·5	0·2 ± 0·1
10 min	12·3 ± 0·8	16·4 ± 2·3	2·0 ± 0·04	2·4 ± 0·1
20 min	18·4 ± 1·1	6·8 ± 2·4	1·1 ± 0·3	3·3 ± 0·9
30 min	25·9 ± 3·6	5·1 ± 1·7	0·4 ± 0·1	3·3 ± 0·4
1 hr	23·6 ± 2·9	0·7 ± 0·07	0·4 ± 0·04	1·7 ± 0·5
2 hr	27·5 ± 3·7	0·3 ± 0·01	0·3 ± 0·04	3·3 ± 0·2
24 hr	8·7 ± 1·6	0·1 ± 0·01	0·2 ± 0·05	0·5 ± 0·1
48 hr	ND†	ND	ND	ND

*dpm × 10^4/gall bladder.

†ND = Not detected. Values were not significantly different ($P > 0·05$ by one-way analysis of variance) from values for corresponding tissue homogenates from control animals.

Values are means ± S.E.M. for groups of three animals. Radioactivity measurements for each tissue sample were made in duplicate.

Table 4. *Distribution of radioactivity in the lung, spleen and testes of mice following iv injection of a single dose of [^{14}C]carmoisine (200 mg/kg; 0·7 μCi)*

Time after dosing	^{14}C-activity (dpm × 10^4/g) in the		
	Lung	Testes	Spleen
5 min	48·0 ± 8·3	4·0 ± 0·2	13·0 ± 2·1
10 min	21·7 ± 3·9	3·8 ± 0·2	7·4 ± 0·7
20 min	18·5 ± 2·7	4·1 ± 0·6	7·0 ± 2·8
30 min	6·6 ± 2·3	3·0 ± 0·5	1·5 ± 0·4
1 hr	1·6 ± 0·4	1·3 ± 0·1	0·9 ± 0·1
2 hr	2·8 ± 1·9	0·6 ± 0·1	0·9 ± 0·1
24 hr	ND*	ND	ND
48 hr	ND	ND	ND

*ND = Not detected. Values were not significantly different ($P > 0·05$ by one-way analysis of variance) from values for corresponding tissue homogenates from control animals.

Values are means ± S.E.M. for groups of three animals. Radioactivity measurements for each tissue sample were made in duplicate.

Table 5. *Excretion of radioactivity in the faeces and urine of mice after administration of a single oral dose of [^{14}C]carmoisine (200 mg/kg; 6·0 μCi)*

Time after dosing (hr)	No. of animals	No. of experiments	Percentage of administered radioactive dose recovered in			
			Faeces*	Urine*	Cage wash	Total
4	7	2	5	4	—	9
8	10	5	22 ± 6	10 ± 4	2·7	35
16	13	5	49 ± 12	17 ± 4	4·5	71
32	15	5	74 ± 8	19 ± 3	4·7	98
64	15	5	64 ± 8	22 ± 4	1·7	88†
96	12	4	64 ± 10	24 ± 4	1·3	89†

*Values are means ± S.E.M. for results from the no. of experiments and animals indicated.
†Not significantly different ($P > 0·05$ by one-way analysis of variance) from the 32-hr value.
Radioactivity measurements for each sample were made in duplicate.

Table 6. *Excretion of radioactivity in the faeces and urine of mice following iv injection of a single dose of [^{14}C]carmoisine (200 mg/kg; 0·7 μCi)*

Time after dosing (hr)	Amount* (percentage) of the administered radioactive dose recovered in		Total recovered (%)
	Faeces	Urine	
24	96 ± 16 (64)	18 ± 0·09 (12)	76
48	138 ± 15 (92)	24 ± 6 (16)	108

*Values are means (dpm × 10^4) ± S.E.M. for groups of three animals. Radioactivity measurements for each sample were made in duplicate.

of elimination (13·9 hr) calculated from the plasma radioactivity decay curves.

A poor reabsorption of carmoisine and/or its metabolites from the intestine is also suggested from the high recovery (92%) of radioactivity in the faeces in the iv experiment. This seems to be confirmed by the low plasma radioactivity levels found when [^{14}C]carmoisine is administered orally to mice.

A preferential excretion into the faeces in rats has been reported for naphthionic acid (Pritchard, Holmes & Kirschman, 1976; Radomski & Mellinger, 1962; Ruddick, Craig, Stavric, Willes & Collins, 1979). The kinetics of this acid after oral administration to rats of a dose of 200 mg FD & C Red No. 2/kg body weight was shown to be similar to that observed when naphthionic itself was given orally (Pritchard *et al.* 1976). These results have led to the conclusion that FD & C Red No. 2 is actively metabolized in the gastro-intestinal tract with the formation of naphthionic acid. Carmoisine metabolism has not been reported yet, but if carmoisine too is actively metabolized it is likely to give rise to the same acid. Data reported here may be an indication of carmoisine metabolism.

Comparison between carmoisine kinetics with that of other azo dyes of similar structure reported by others (Radomski & Mellinger, 1962; Ruddick *et al.* 1979) is difficult at present because different animal species and sexes were used. Nevertheless the radioactivity decay curves after oral administration of [^{14}C]carmoisine to mice show a profile similar to that found in the rat for naphthionic acid originating from azo dyes.

Work is in progress on the evaluation of radioactivity associated with the unchanged dye and with its metabolites after [^{14}C]carmoisine administration.

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COMPARATIVE MUTAGENICITY OF TWO TRIARYLMETHANE FOOD DYES IN SALMONELLA, SACCHAROMYCES AND DROSOPHILA

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Abstract—The dye Violet BNP C.I. 42581 (C.I. Food Violet 3), as used in food, was shown to induce point mutations in *Salmonella typhimurium*, mitotic convertants and recombinants in *Saccharomyces cerevisiae* and recessive X-linked lethal mutants in *Drosophila melanogaster*. One or more constituents of Violet BNP may have been genotoxic, as the sample of dye used was shown to be an impure mixture, consisting of at least seven different chemical components. A second dye, Lissamine Green B C.I. 44090 (Wool Green 5, Acid Green 50, Green 5) was weakly mutagenic when tested with Salmonella. However, no conclusive evidence of genetic activity in yeast was found, and in two experiments no lethal mutants were detected in Drosophila.

INTRODUCTION

Short-term mutagenicity testing has been proposed as a means of screening chemical substances for potentially hazardous effects (IRLG, 1979; Bridges, 1976a; DHEW, 1977) and in a number of cases has led to the identification of chemicals with carcinogenic potential (McCann & Ames, 1977). In particular, the concurrent use of different test systems has been advocated (Bridges, 1976b; Stoltz, Poirier, Irving, Stich, Weisburger & Grice, 1974) as the most promising way to ensure that potent carcinogens that present a high potential risk to man do not escape the screening process.

In a separate report we gave details of the mutagenicity testing of a large number of triarylmethane dyes using *Salmonella typhimurium* (Bonin, Baker & Farquharson, 1981). Two of these, the more potent mutagen, Violet BNP, and the weak mutagen, Lissamine Green B, have been examined in more detail here. The dyes were assayed in yeast and Drosophila to determine the extent of agreement between the three short-term test methods.

EXPERIMENTAL

Salmonella/mammalian microsome assay. Structures of Violet BNP (Pointing Ltd, Prudhoe, UK) and Lissamine Green B (Aldrich Chemical Company, Milwaukee, WI, USA) are given in Fig. 1. The dyes were dissolved in dimethylsulphoxide at various concentrations and the *Salmonella typhimurium* mutagenesis assay was then carried out as previously described (Bonin & Baker, 1980). Briefly, 100 μl bacterial broth culture, 100 μl aliquots of dye solution and 500 μl S9 mix (as required) were incorporated in 2 ml top agar. This was mixed and plated over 28 ml of minimal agar containing trace quantities of histidine and biotin. The S9 mix was prepared from liver microsomes and soluble enzyme fraction (Ames, McCann & Yamasaki, 1975).

Saccharomyces D7 mutagenesis assays. Cells of *Saccharomyces cerevisiae* strain D7, harvested from log-phase cultures grown on glucose medium at 30°C, were resuspended in phosphate buffer pH 7·0, at a concentration of 3×10^7 cells/ml. Each dye solution was added in required amounts to these cell suspensions which were then incubated at 37°C for 1 hr. These conditions are optimal for activation of chemicals by yeast cells (Callen & Philpot, 1977). Treatments were ended by diluting and washing cells in ice-cold buffer. Appropriate dilutions were plated on media to select for *trp* 5 and *ade* 2 convertants and to allow estimation of survival and frequency of *ade* 2 mitotic recombinants (Zimmerman, Kern & Rasenberger, 1975). Uptake of Violet BNP by yeast cells was determined by incubating log or stationary phase cells for 1 hr at 37°C with 0·13 M dye. Cells were washed three times with ice-cold buffer and the optical density (OD) of resuspended cells was measured at 600 nm.

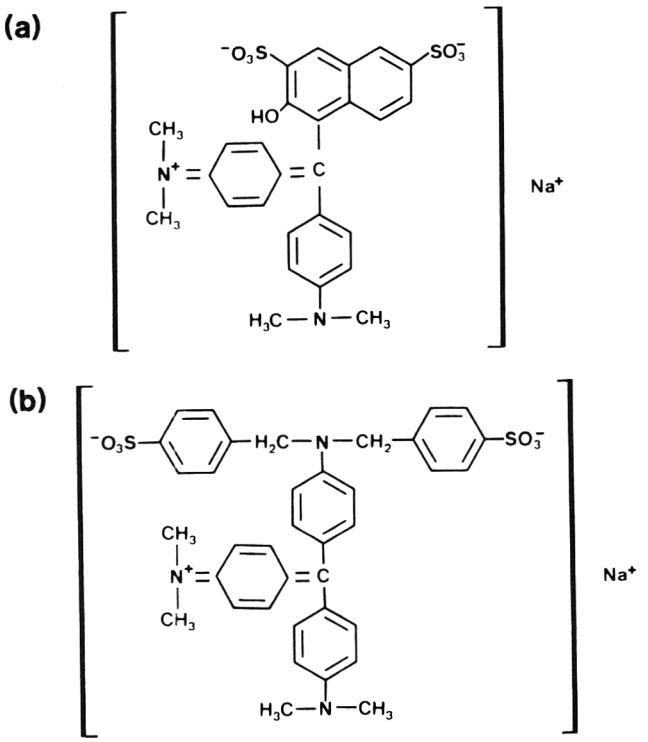

Fig. 1. Structural formulae of the food dyes (a) Lissamine Green B and (b) Violet BNP.

Drosophila sex-linked recessive lethal assay. The frequency of sex-linked recessive lethal mutations in *Drosophila melanogaster* was estimated by the *Basc* method (Vogel & Sobels, 1976). Mutants were scored by mating 12 treated 4–7 day old Canton-S males to *Basc* females, three females for each male, with successive broods from fresh virgin females at 72-hr intervals. All cultures were maintained at 25°C and suspected lethals were checked in the F3 generation. In different experiments, adult male flies were either fed for 72 hr on dye solution dissolved in 5% sucrose, or were injected with dye in 0·7% NaCl solution. Alternatively Drosophila larval stages were reared on standard semolina-treacle-agar culture medium containing dye. The positive control for these experiments was ethylmethane sulphonate (EMS), adults having been fed for 72 hr and third instar larvae for 48 hr on a yeasted suspension containing EMS.

High-pressure liquid chromatography (HPLC). A Hewlett-Packard 1084 liquid chromatograph fitted with a reverse phase column (79918A) was used for HPLC analysis. For Violet BNP the solvent gradient was methanol–water (1:9; v/v) to methanol–water (9:1, v/v) in 30 min, while for Lissamine Green B the solvent gradient was methanol–water (1:9; v/v) to methanol–water (4:6; v/v) in 25 min and increasing to methanol–water (9:1; v/v) over the next 5 min to give a total run-time of 30 min. The tracing for Lissamine Green B was at 600 nm and for Violet BNP, 585 nm. The injection volume in each case was 0·02 ml of a 0·5 mg/ml solution in methanol.

RESULTS

Mutagenesis tests

Table 1 lists results of tests for frameshift or base-pair substitution mutations at the *his* loci in *Salmonella typhimurium.* Both Violet BNP and Lissamine Green B give evidence of mutagenicity in strains TA1538 and TA98 in the presence of S9. Although the reproducibility of the Lissamine Green B result was verified using strain TA98, it was found that a significant doubling of revertant colonies only occurs at dye concentrations of $3{\cdot}2 \times 10^{-4}$ M or more ($120 \pm 7{\cdot}4$ revertants; n = 10) as compared with control plates ($60 \pm 3{\cdot}7$ revertants; n = 10). This food colouring is clearly less mutagenic than Violet BNP in Salmonella.

Different samples of the two dyes gave different results. Another sample of Lissamine Green B, obtained from a different source (Hodgsons Dye Agencies, Sydney) was not mutagenic in Salmonella. Violet BNP obtained from the same source 18 months earlier was mutagenic but with 25% of the potency of the sample tested in this study, as judged by the number of revertant colonies at dye concentrations of $4{\cdot}66 \times 10^{-5}$ M, and when tested at the same time as the present sample.

In *Saccharomyces cerevisiae* Violet BNP also induced *trp* 5 convertants and *ade* 2 mitotic recombinants when incubated with log-phase cells of the D7 strain for 1 hr at 37°C (Table 2). Incubation of Violet BNP with stationary phase cells at 30°C or 37°C for periods up to 24 hr did not result in any increase in conversion or mitotic recombination when compared with controls (results not given). In addition, Violet BNP was not genetically active in cells from stationary phase cultures incubated together with an *in vitro* activation system using the 9000 g supernatant fraction of phenobarbital- or 3-methylcholanthrene-induced rat liver (results not given). Dye uptake

Table 1. *Point mutations induced in Salmonella*

Dye concn, M and (μg/plate)	With or without S9 mix...	No. of *his*$^+$ revertant colonies/plate using *S. typhimurium* strain...									
		TA98		TA100		TA1535		TA1537		TA1538	
		−	+	−	+	−	+	−	+	−	+
Lissamine Green B*											
0		27	49	190	138	37	25	12	9	23	36
$1{\cdot}85 \times 10^{-6}$(32)		36	47	158	143	27	12	8	10	19	36
$5{\cdot}78 \times 10^{-6}$(100)		22	47	147	117	36	13	9	11	18	41
$1{\cdot}85 \times 10^{-5}$(320)		26	47	154	122	45	15	8	8	20	41
$5{\cdot}78 \times 10^{-5}$(1000)		19	77	143	129	38	10	10	14	18	54
Violet BNP											
0		23	34	157	137	21	16	7	9	17	25
$1{\cdot}49 \times 10^{-6}$(32)		16	54	104	110	22	12	4	6	13	45
$4{\cdot}66 \times 10^{-6}$(100)		20	76	115	123	18	13	9	12	11	66
$1{\cdot}49 \times 10^{-5}$(320)		15	197	132	124	16	12	12	11	13	178
$4{\cdot}66 \times 10^{-5}$(1000)		14	706	70	118	8	4	7	21	2	427

Values are means of two plates; those underlined are considered to indicate an increase in revertant colonies by comparison with controls.

*In a separate experiment $3{\cdot}2 \times 10^{-4}$ M Lissamine Green B doubled the background mutation rate of strain TA98 (Bonin *et al.* 1981).

studies were conducted to investigate the possibility of preferential absorption of Violet BNP by log-phase cells. After incubation of cells with the dye, the increase in OD of log-phase cells (0·75 OD units/10^7 cells) was 50% greater than stationary phase cells (0·036 OD units/10^7 cells).

Tests with Lissamine Green B in yeast indicate a similar cytotoxic effect to that of Violet BNP (Table 2). A positive mutagenic response was found at the highest concentration tested. However, at this concentration, survival was low (8%), there were no induced *ade* 2 mitotic recombinants and no overall dose response was evident. It is therefore considered that evidence for genetic activity of Lissamine Green B in yeast is inconclusive.

Adult male Drosophila were insensitive to any mutagenic effect of Violet BNP at $1{\cdot}2 \times 10^{-3}$ M concentration, regardless of whether the dye was fed to the flies or whether it was injected into the abdomen (Table 3). However, adults fed $2{\cdot}5 \times 10^{-2}$ M dye gave a significant number of mutations. By contrast, larvae elicited a definite mutagenic response even when fed on as little as 5×10^{-5} M dye.

No evidence of recessive lethal mutations was found among the first brood progeny of either adults (0/240) or larvae (0/94) treated with $2{\cdot}5 \times 10^{-3}$ M or $2{\cdot}5 \times 10^{-2}$ M Lissamine Green B, respectively.

Chemical purity of dyes

The two food dyes were examined for purity by HPLC (Fig. 2). At 600 nm, Lissamine Green B gave a trace indicating the presence of two significant components, with the area of the major peak representing 89% of the total area. The trace for Violet BNP indicates at least seven significant components absorbing at 585 nm, with the two major peaks representing 35·2% and 22·9% of the total area, accompanied by numerous other minor components. The small peaks in the first few minutes of the profile arise from impurities present in the water used for the solvent gradient.

Table 2. *Gene convertants and mitotic recombinants in yeast strain D7*

Dye concn (M)	Survival (%)	*Trp 5* convertants (frequency/ 10^4 survivors)	*Ade* 2 mitotic recombinations (frequency/ 10^4 survivors)	Total genetically altered colonies at *ade* 2 locus* (frequency/10^4 survivors)
Lissamine Green B				
0	100	0·14	−†	16·6
$2{\cdot}77 \times 10^{-5}$	100	0·16	−†	10·8
$1{\cdot}21 \times 10^{-4}$	79	0·19	2·3	11·5
$5{\cdot}72 \times 10^{-4}$	72	0·16	−†	12·7
$2{\cdot}84 \times 10^{-3}$	8	0·51	−†	82·8
Violet BNP				
0	100	0·11	−†	7·7
$1{\cdot}84 \times 10^{-5}$	79	0·13	−†	11·6
$7{\cdot}67 \times 10^{-5}$	87	0·32	3·4	8·4
$3{\cdot}84 \times 10^{-4}$	82	0·41	5·4	32·4
$1{\cdot}9 \times 10^{-3}$	26	1·47	22·8	109·4

*Includes *ade* 2 convertants and mitotic recombinants.

†No *ade* 2 mitotic recombinants (twin spot colonies) observed among colonies.

Table 3. *Sex-linked recessive lethals induced in Drosophila by Violet BNP*

Experiment no.	Stage	Sex-linked recessive lethals				Mutation frequency (%)
		Brood			Total	
		A	B	C		
1*	Adult	0/254	0/221	0/293	0/768	<0·1%
3*	Adult	0/230	1/303	0/279	1/812	0·1%
5†	Adult	2/302	0/315	0/318	2/935	0·2%
2‡	Larval	2/271	1/219	3/218	6/708	0·9%
4‡	Larval	3/245	2/276	2/197	7/718	1·0%
6§	Adult	6/190	—	—	6/190	3·2%
7§	Larval	18/186	—	—	18/186	9·7%
Negative controls						
−1‖	—	0/444	1/321	0/299	1/1064	0·1%
−2‖	—	0/313	0/395	0/386	0/1094	<0·1%
−3‖	—	0/280	—	—	0/280	<0·1%
Positive controls						
+1	Adult	60/180	—	—	60/180	33%
+2	Larval	1/122	—	—	1/122	0·8%

*Adult males were fed on $1{\cdot}2 \times 10^{-3}$ M-dye solution in 5% sucrose for 72 hr.
†Adult males were injected with 0·1 μl $1{\cdot}2 \times 10^{-3}$ M-dye in 0·7% NaCl solution.
‡Larval stages were reared on semolina-treacle-agar culture medium containing 5×10^{-5} M dye.
§Adults or larvae were fed on $2{\cdot}5 \times 10^{-2}$ M dye for 72 and 48 hr respectively.
‖The first two negative control runs were separated by an interval of 6 months.
¶Positive control adults were fed $2{\cdot}5 \times 10^{-2}$ M-ethyl methane sulphonate (EMS) and third instar larvae were fed on a yeasted suspension containing $2{\cdot}5 \times 10^{-3}$ M-EMS.

DISCUSSION

Previous investigations of yeast mutagenesis have shown that log-phase cells can activate various promutagens and that this is dependent on the activity of cytochrome *P*-450 mediated monooxygenase enzymes in the yeast cells (Callen & Philpot, 1977). However, other variable factors may account for differences between actively growing and stationary cultures. For example, the absence of genetic activity of Violet BNP in stationary cells, in the presence of an *in vitro* mammalian liver activation system, may be due to a reduced permeability of stationary cells. In this study, spectrophotometric measurements indicated that log-phase cells were capable of absorbing 50% more Violet BNP per cell than cells harvested from stationary-phase cultures.

In Drosophila, the different sensitivities of adult and larval stages may be related to the intense metabolic activity of the larval fat body. This may be able to achieve activation of the dye to a mutagenic metabolite not readily formed in the adult. Since there was no evidence of clustering of lethals within particular treated males, it seems that the dye acts on post-meiotic stages in the late third instar. It is also possible that the larval response is related to a transitory period of susceptibility to the mutagen rather than to overall activity of the fat body, for, Auerbach & Moser (1953) showed that when formaldehyde was fed to Drosophila, mutagenicity occurred only in the auxocyte stage in spermatogenesis i.e. the early spermatocytes; no mutagenic response was obtained by feeding formaldehyde to adult flies.

Although the results of the Salmonella, Saccharomyces and Drosophila tests are qualitatively in agreement, indicating that Violet BNP is, or contains, a mutagen and that Lissamine Green B is not mutagenic, or only marginally so, considerable inter-test variability is seen when comparing dye potency in each case. In Salmonella there is a 27–30 fold difference between dye potencies (in revertants/nM) and in yeast there is a 2·3–5·5 fold difference (convertants or altered colonies/10^4 survivors/nM). These potency differences may simply reflect the differences in mutagenic mechanisms in Salmonella and Saccharomyces. Nevertheless, the results do highlight the difficulty in interpreting 'potency' in mutagenesis assays.

The same or a similar molecular species may be responsible for mutagenicity of these dyes, particularly since other arylmethane dyes, Benzyl Violet 4B C.I. 42640, Guinea Green B C.I. 42085, Light Green SF C.I. 42095 and basic fuchsin C.I. 42500–42510 are active in the same Salmonella strains and also in the presence of S9 (Bonin *et al.* 1981). As with Violet BNP and Lissamine Green B, different samples of other arylmethane dyes also varied in their mutagenicity towards Salmonella, suggesting that variations in impurities may be responsible. Violet BNP, as used commercially in food, was shown in this study to be a mixture, containing a considerable number of components, any one of which may be responsible for the genotoxic effect. In the case of Lissamine Green B, the HPLC results indicate that any mutagenic component was probably only a trace contaminant.

In view of the mutagenicity of Violet BNP in three different organisms, in the presence of both *in vitro* and *in vivo* metabolic activation systems, there is a need for reappraisal of this dye for use in food, cosmetics or drugs. The finding that mutagenicity in arylmethane dyes may be related to impurities demonstrates the need to identify minor components of these compounds and to determine their possible contribution to any toxicological effects.

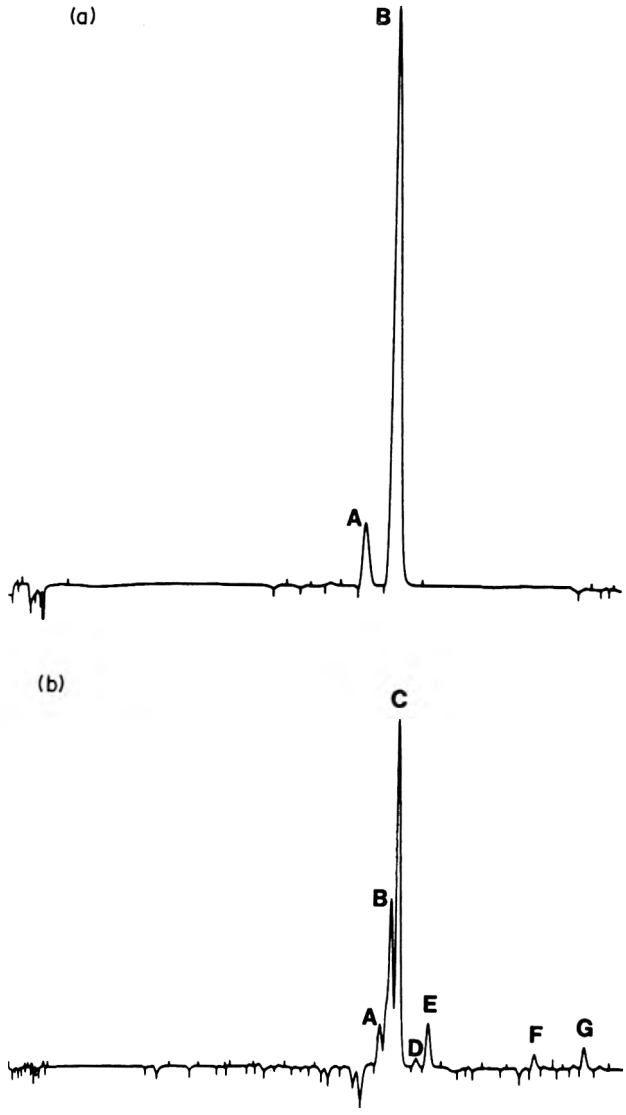

Fig. 2. HPLC profiles of (a) Lissamine Green B at 600 nm and (b) Violet BNP at 585 nm. For Lissamine Green B, peaks A and B occurred at 17·4 and 18·93 min respectively, and for Violet BNP, peaks A-G occurred at 18·05, 18·57, 18·92, 19·83, 20·43, 25·66 and 28·1 min respectively.

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INHIBITION OF *N*-NITROSAMINE FORMATION BY SOYA PRODUCTS

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Abstract—Soya products such as Tôfu (soya-bean curd), soya milk, Miso (soya-bean paste) and Bonlact (powdered soya milk with additives) are very common foods commercially available in Japan. The effects of these soya products on nitrite concentration and on the formation of *N*-nitrosamines *in vitro* were investigated. All of these soya products effectively decreased nitrite levels at pH 3 and 4, the effect being greater at pH 3. The soya products inhibited the formation of *N*-nitrosodimethylamine and *N*-nitrosodiethylamine from sodium nitrite and the appropriate dialkylamine at gastric pH. For example, 20% (w/v) Tôfu, 60% (v/v) soya milk, 10% (w/v) Miso and 4% (w/v) Bonlact inhibited by 46, 51, 74 and 44%, respectively, the formation of *N*-nitrosodimethylamine from 0·2 M-nitrite and 0·05 M-dimethylamine at pH 3 and 37°C for 3 hr. The loss of nitrite and inhibition of *N*-nitrosamine formation by soya products may be attributed to the lipids or to the minor phenolics contained in the products.

INTRODUCTION

Under gastric conditions reactions between nitrite and secondary amines can produce *N*-nitrosamines (Mirvish, 1970; Sander & Seif, 1969), which are potential human carcinogens (Druckrey, Preussmann, Ivankovic & Schmähl, 1967). Nitrite may be readily derived by bacterial reduction (Ayanaba & Alexander, 1973) and salivary reduction (Ishiwata, Boriboon, Nakamura, Harada, Tanimura & Ishidate, 1975; Spiegelhalder, Eisenbrand & Preussmann, 1976; Tannenbaum, Weisman & Fett, 1976) from the nitrate present in a wide variety of vegetables (Walker, 1975; Yanagihara, Komoda, Yoneyama & Yamada, 1963). Nitrite and nitrate are also synthesized endogenously by nitrifying bacteria in the human body (Tannenbaum, Fett, Young, Land & Bruce, 1978).

Several foodstuffs are known to decrease nitrite and to exert inhibitory effects on the formation of nitrosamines under gastric conditions. These include cows' milk (Kurechi & Kikugawa, 1979) and Japanese radish (*Raphanus sativus*), a common vegetable in Japan (Kurechi, Kikugawa & Fukuda, 1980a). Alcoholic drinks such as whisky, wine and Japanese *sake*, decreased nitrite levels at pH 3 and pH 5, inhibited the formation of *N*-nitrosamines at pH 3, but enhanced it at pH 5 (Kurechi, Kikugawa & Kato, 1980c). In this study, the effects of soya products, very common foods in Japan, on nitrite levels and the formation of the *N*-nitrosamines were investigated. The soya products studied were soya-bean curd (called "Tôfu" in Japan), soya milk, soya-bean paste (called "Miso" in Japan) and Bonlact (powdered soya milk with additives).

EXPERIMENTAL

Materials. Tôfu (soya-bean curd) and Miso (soya-bean paste) were obtained from a local market in Tokyo. Fresh soya milk with no additives was obtained from a soya-bean curd manufacturer. Bonlact (trade name) produced by Wakodo Company Ltd, Tokyo, is a powdered soya milk with additives (a large amount of linoleic acid and a small amount of vitamins and minerals). The constituents (%, w/v) of Tôfu, soya milk and Miso, given by the Science Technology Agency in Japan, were as follows: Tôfu—protein, 4·9; lipid, 2·8; carbohydrate, 1·5; ash, 1·1; soya milk—protein, 3·6; lipid, 2·0; carbohydrate, 2·9; fibre, 0·2; ash, 0·5; Miso—protein, 16·8; lipid, 6·9; carbohydrate, 13·6; fibre, 2·2; ash (NaCl), 13. Bonlact contained 22·3% (w/v) protein, 18% lipid (7% being added linoleic acid), 52·9% carbohydrate 1·0% fibre and 3·8% ash. All of the soya products were divided as finely as possible before use. Other materials used in the experiments were the same as described previously (Kurechi & Kikugawa, 1979).

Analytical methods. A Hitachi 101 spectrophotometer was used for the spectrophotometric assay of nitrite. Griess reagent was prepared by mixing, just before use, equal volumes of 1·0% (w/v) sulphanilic acid in 30% acetic acid and 1·0% (w/v) 1-naphthylamine in 30% acetic acid.

A Yanaco G80 gas chromatograph, equipped with a hydrogen flame ionization detector and a glass column (3 mm ID × 2 m) packed with polyethylene glycol 6000 (25%) on 80–100 mesh Chromosorb W AW, was used to determine the nitrosamines. The chromatograph was operated isothermally at 120°C (column temperature) and at 140°C (injection and detector temperature) with a nitrogen carrier-gas flow of 25 ml/min. The amount of *N*-nitrosamine in the extract was determined by comparing the peak area of the samples (5 μl) with that of the authentic standard solution in chloroform (5 μl of 0·40 mg/ml).

Nitrite determination. Reaction mixtures, of a total volume of 50 ml, were made up by adding known amounts of each of the soya product samples to 35–45 ml 0·2 M-sodium citrate–HCl buffer (pH 3·0 or 4·0) and 5·0 ml 1·0 mM-$NaNO_2$. The reaction mixtures were put into stoppered tubes and incubated at 37°C for various periods of time. After incubation, the reaction mixture was filtered through filter paper and then through a 0·45 μm membrane filter (Toyo Roshi Co.,

Table 1. *Percentage decreases in nitrite levels in reaction mixtures of soya products and nitrite incubated at 37°C for 120 min*

Treatment		
Soya product (concn in reaction mixture)	pH	Decrease in nitrite level (%)*
Tôfu (12%, w/v)	3·0	71
	4·0	24
Soya milk (20%, v/v)	3·0	70
	4·0	32
Miso (1·4%, w/v)	3·0	76
	4·0	35
Bonlact (2%, w/v)	3·0	58
	4·0	32

*Initially the reaction mixture was 0·1 mM with respect to $NaNO_2$.

Ltd, Tokyo). From the filtrates, 1-ml portions were removed and diluted with 5·0 ml water containing 0·10 ml Griess reagent. The mixtures were kept at room temperature for 15 min and then the absorbances at 520 nm were measured (Kurechi, Kikugawa & Kato, 1979). Each experiment was carried out at least three times, and representative results from experiments carried out simultaneously are shown in Table 1 and Fig. 1.

N-Nitrosamine determination. Various amounts of each of the soya products were added to mixtures containing 5·0 ml 2 M-$NaNO_2$, 2·5 ml M-dimethylamine hydrochloride or 2·5 ml M-diethylamine hydrochloride and 25 ml 0·2 M-sodium citrate-HCl buffer of the desired pH. The effects of Tôfu and Miso on *N*-nitrosamine production were also tested in mixtures containing 5 ml 0·5 M-$NaNO_2$, 2·5 ml 2 M-dimethylamine and 25 ml 0·2 M-sodium citrate–HCl buffer at pH 3. The reaction mixtures were adjusted to the required pH with a small amount of concentrated hydrochloric acid and then made up to 50 ml with water. They were incubated in stoppered flasks at 37°C for 3 hr. Portions of 10 ml were mixed with 2·0 g NaCl and 5 ml 5 N-NaOH and extracted with 40 ml chloroform. The nitrosamine content in the chloroform extracts was determined as has been previously reported (Kurechi *et al.* 1980a,c). The recoveries of *N*-nitrosamine standards by chloroform in the presence of food products were almost quantitative. Each experiment was carried out at least three times, and representative results are shown in Table 2 and Figs 1 & 2 as a percentage inhibition of *N*-nitrosamine formation relative to a control reaction mixture to which no soya product was added.

RESULTS

Decreases in nitrite levels due to reaction between soya products and nitrite

Decreases in nitrite concentration due to reactions with soya products in aqueous suspension were measured in terms of the production of azo-dye with Griess reagent. Control solutions without any added soya sample lost negligible amounts of nitrite during incubation for up to 2 hr. The filtrates from a sample solution to which nitrite was not added had negligible absorbance at 520 nm, thus indicating that there was very little interference of materials extracted from the food products in the reaction used to detect nitrite. Figure 1 shows the representative time course of the loss of nitrite in mixtures of 0·1 mM-$NaNO_2$ and 6, 12 or 20% (w/v) of Tôfu incubated at 37°C in buffer at pH 3 or 4. The rate of loss of nitrite was faster at pH 3 than at pH 4 and at pH 3 was dependent on the amount of Tôfu present. The rate of the loss varied somewhat from preparation to preparation, because of variations in water content and the difficulty of dividing up the soya-bean curd to the same degree of fineness each time.

Soya milk, Miso and Bonlact similarly decreased nitrite under the same conditions. The percentage losses of nitrite in mixtures of 0·1 mM-$NaNO_2$ and each of soya products incubated at 37°C for 120 min are shown in Table 1. With all of the soya products, the losses of nitrite at pH 3 were greater than at pH 4. The values for nitrite loss varied slightly from preparation to preparation of each product. It may be concluded that the potency of nitrite loss per g wet or dry weight of soya product was in the following order: Miso > Bonlact > Tôfu > soya milk.

Effects of soya products on N-nitrosamine formation

The effects of these soya products on the formation of *N*-nitrosamines by reaction between nitrite and dimethylamine or diethylamine under mild acidic conditions (pH 3, 4 and 5) were investigated. Figure 2 shows the representative profiles of the effects of 10 and 20% (w/v) Tôfu and 10% (w/v) Miso on *N*-nitrosodimethylamine (NDMA) formation by reaction of 0·2 M-nitrite with 0·05 M-dimethylamine at 37°C for 3 hr. Tôfu and Miso inhibited NDMA formation at each pH, the inhibition being most apparent at pH 3. Figure 3 shows the representative profiles of the effects of 20% (w/v) Tôfu and 10% (w/v) Miso on *N*-nitrosodiethylamine (NDEA) formation by reaction of nitrite and diethylamine. Both soya-bean products considerably inhibited NDEA formation at pH 3 and 4. Tôfu and Miso were also effective in inhibiting NDMA formation by reaction of 0·05 M-nitrite with

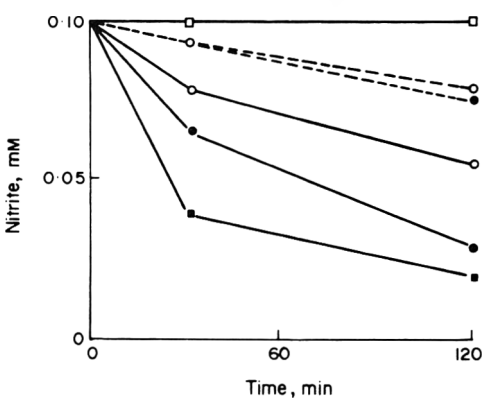

Fig. 1. Decrease in nitrite concentrations in mixtures of 0·1 mM-$NaNO_2$ with 0 (□), 6 (○), 12 (●) or 20 (■) % (w/v) Tôfu incubated at pH 3·0 (——) or 4·0 (---) in citrate buffer at 37°C.

Table 2. *Effects of soya products on the formation of NDMA and NDEA from nitrite and the appropriate dialkylamine* in vitro

Soya product	Concn in reaction mixture	Inhibition (%) of formation of NDMA	Inhibition (%) of formation of NDEA
Reaction mixture A*			
None	—	0	0
Tófu	10% (w/v)	28	ND
	20% (w/v)	46	42
Soya milk	30% (v/v)	44	ND
	60% (v/v)	51	ND
Miso	5% (w/v)	36	ND
	10% (w/v)	74	74
Bonlact	2% (w/v)	38	ND
	4% (w/v)	44	ND
Reaction mixture B†			
None	—	0	0
Tófu	10% (w/v)	5	ND
	20% (w/v)	17	ND
Miso	5% (w/v)	43	ND
	10% (w/v)	78	ND

NDMA = *N*-Nitrosodimethylamine
NDEA = *N*-Nitrosodiethylamine
ND = Not determined

*N-Nitrosamine formation from 0·2 M-$NaNO_2$ and 0·05 M-dimethylamine or 0·05 M-diethylamine at pH 3·0 and 37°C for 3 hr.

†NDMA formation from 0·05 M-$NaNO_2$ and 0·10 M-dimethylamine at pH 3·0 and 37°C for 5 hr.

0·1 M-dimethylamine at 37°C for 5 hr (Table 2). Soya milk and Bonlact also inhibited the formation of NDMA and NDEA by reaction of 0·2 M-nitrite with 0·05 M-dimethylamine and 0·05 M-diethylamine, respectively. The inhibitory effects of each of the soya products on *N*-nitrosamine formation at pH 3 are summarized in Table 2. All of the products showed a concentration-dependent inhibition of *N*-nitrosamine formation. The degree of inhibition varied slightly between samples, probably because of the different water contents of the preparations. It seemed that the potency of inhibition per g wet or dry weight of soya

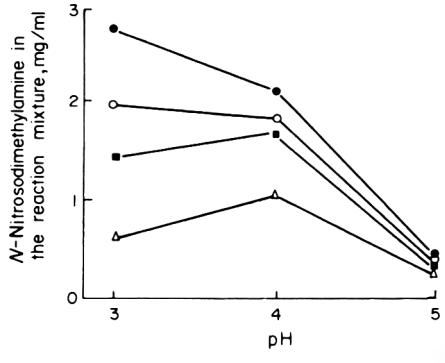

Fig. 2. Effects of 0 (●), 10 (○) or 20 (■) % (w/v) Tófu or 10% (w/v) Miso (△) on *N*-nitrosodimethylamine formation from 0·2 M-$NaNO_2$ and 0·05 M-dimethylamine in a 3-hr incubation at 37°C and pH 3, 4 or 5.

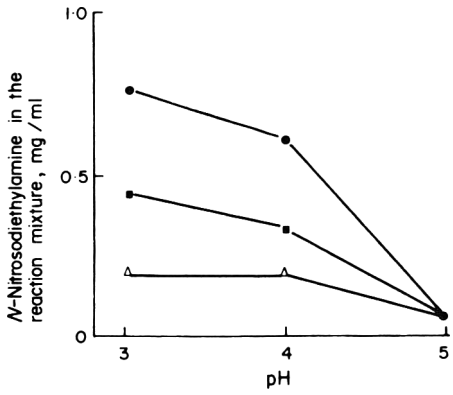

Fig. 3. Effects of no soya product addition (●), of 20% (w/v) Tófu (■) and of 10% (w/v) Miso (△) on NDEA formation from 0·2 M-$NaNO_2$ and 0·05 M-diethylamine in a 3-hr incubation at 37°C and at pH 3, 4 or 5.

products was in the following order: Bonlact > Miso > Tófu > soya milk.

DISCUSSION

The present experiments showed that soya products effectively reduced nitrite and inhibited the formation of NDMA *in vitro* under mild acidic conditions. Although a reduction in nitrite concentration may not be the sole cause of inhibition of the *N*-nitrosamine formation, the soya products such as Bonlact and Miso that reduced more nitrite were more effective inhibitors of *N*-nitrosamine formation. The soya products may inhibit *N*-nitrosamine formation in the stomach in which nitrite and secondary amines are simultaneously present. Tófu is a common food in Japan. Miso is frequently used in Japan as a flavouring for soup (called "Misosiru"), and the usual Miso content of the soup is about 5–10%—the same level as was present in the incubation mixtures in the present experiments. Soya milk is also a common drink in Japan, and Bonlact is sold in Japan as a nutrient feed for infants.

All of the soya products contain proteins, lipids, carbohydrates and other minor nutrients, but the contents vary from product to product. The substance(s) that reduced nitrite and inhibited *N*-nitrosamine formation have not yet been elucidated. In our previous report (Kurechi & Kikugawa, 1979), unsaturated fatty acids such as linoleic and oleic acids, and a fat emulsion for infusion therapy (Intralipid) composed of soya-bean oil and yolk lecithin effectively reduced nitrite and subsequently inhibited nitrosamine formation. All of the soya products investigated in this study contain much lipid, and the products with the highest lipid contents such as Bonlact and Miso showed the greatest effects. Thus, the unsaturated fatty acid components of the lipids present in the products might be responsible for the reduction of nitrite and the inhibition of *N*-nitrosamine formation. Furthermore, soya beans contain many phenolic compounds (Sosulski, 1979), and it has been demonstrated that a variety of phenolics are effective in inhibiting *N*-nitrosamine formation (Gray & Dugan, 1975; Kurechi *et al.* 1979; Kurechi, Kikugawa & Kato, 1980b;

Pensabene, Fiddler, Mergens & Wasserman, 1978; Yamada, Yamamoto & Tanimura, 1978). Thus, certain phenolics in soya products might have also contributed to the reduction of nitrite and the inhibition of nitrosamine formation.

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THE INFLUENCE OF MILK IN THE DIET ON THE TOXICITY OF ORALLY INGESTED LEAD IN RATS

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Abstract—The influence of milk in the diet on the toxicity of orally ingested lead was investigated in male weanling Wistar rats. Three groups of 20 rats were fed diets based on soya protein, whole milk or lactose-hydrolysed milk, and half of the animals in each group were given 20 μg lead (as lead acetate)/g diet. After 8 wk, biochemical tests for lead toxicity were carried out and tissue-lead levels were measured. While all of the rats given lead-supplemented diets had higher concentrations of lead in bone, brain, blood, kidney and liver than those given diets without added lead, the increase was significantly greater in all tissues in the group given lead in milk. Lead-treated animals fed a milk diet from which lactose had been removed by hydrolysis had tissue-lead concentrations similar to those of rats fed the lead-containing soya diet. Lead supplementation did not increase levels of urinary δ-aminolaevulinic acid (ALA) or free erythrocyte protoporphyrin, but did cause inhibition of erythrocyte ALA dehydratase activity. ALA dehydratase inhibition was not greater in the group given lead in milk despite the fact that tissue lead concentrations were 30–60% higher in these rats, compared to those given lead in soya or in lactose-hydrolysed milk. In a second experiment two groups of rats were fed two meals per day. Both groups were given 5 g soya-based diet containing 60 μg lead/g in the morning. In the evening one group was given, *ad lib.*, the milk diet without added lead, while the other was given, *ad lib.*, the soya diet without added lead. There was no difference in tissue-lead concentrations between the two groups, indicating that milk must be present in the gastro-intestinal tract at the same time as lead in order to increase the levels of lead in the tissues. These studies show that milk consumption increases tissue-lead concentrations in rats fed low concentrations of lead and milk simultaneously. Lactose appears to be the factor in milk responsible for this increase. These studies indicate the need for further research on interactions of diet and lead toxicity and the need for more sensitive methods for assessing the health effects of chronic exposure to low levels of lead.

INTRODUCTION

The extent of the physiological and biochemical changes resulting from exposure to lead in food or the environment depends on the amount of lead ingested and on the proportion of lead that is absorbed and retained in the body tissues. The absorption and retention of lead is influenced by many factors including the species and age of the animal and its nutritional status, the chemical and physical form of the lead, and the composition of the diet.

Dietary factors that influence the susceptibility of animals to lead toxicity are poorly understood (Committee on Toxicology, 1976). However, evidence suggests that dietary factors may have a significant effect on the retention of lead (Barltrop & Khoo, 1975; Stephens & Waldron, 1975). Most of the work reported to date has dealt with the enhancement of lead absorption as a result of increasing or decreasing the quantity of particular dietary components. Diets low in calcium or phosphorus or high in vitamin D enhance lead absorption and retention in various animal species (Mahaffey, 1974; Stephens & Waldron, 1975). Since milk and dairy products represent a major source of calcium and phosphorus in the diet, it might be expected that milk would protect against excess lead absorption. For over a century, milk was recommended as a prophylactic for lead poisoning in industry (Stephens & Waldron, 1975). However, recent studies in young growing rats have shown that milk diets increase the uptake of radioactive lead by the intestine (Kello & Kostial, 1973). In these experiments only the short-term retention of ^{203}Pb was measured and the long-term effects of milk in the diet on body lead burden, tissue-lead distribution or biochemical evidence of lead toxicity were not evaluated. Furthermore the experimental milk-based diets were lower in calcium, phosphorus, vitamin D and iron than were the control diets. Each of these factors could have contributed to the greater uptake of ^{203}Pb by the milk-fed rats. The purpose of the study reported here was to investigate the effect of milk and lactose-hydrolysed milk on the retention of orally ingested lead in rats fed low levels of lead (20 μg/g diet) over a relatively long period of time. The tissue distribution of lead and biochemical indicators of lead toxicity were also investigated.

EXPERIMENTAL

Experiment 1

Three groups of 20 weanling male Wistar rats, weighing 45–50 g were obtained from the University of Western Australia breeding colony. They were fed semi-purified diets based on isolated soya protein,

Table 1. *Composition of the diets fed, with or without added lead, to male Wistar rats for 8 wk*

Constituent	Concn (%*) in Soya diet	Milk diet	Lactose-hydrolysed milk diet
Soya†	21·28	—	—
Dried whole milk	—	76·92	—
Lactose-hydrolysed milk‡	—	—	74·02
Lard	17·0	—	—
Corn oil	2·0	—	—
Cellulose	1·0	1·0	1·0
DL-Methionine	0·3	—	—
Trace mineral mix§	0·029	0·029	0·029
Major minerals‖	5·2295	—	—
Vitamin premix¶	1·0	1·0	1·0
Vitamin D (μg)	6·3	4·15	—
$FeSO_4.7H_2O$ (mg)	27·313	29·85	18·61
Water	3·3	1·92	3·37
$CaCO_3$	—	—	0·098
KH_2PO_4	—	—	0·088
Starch	48·834	19·101	20·376

*Concentrations are expressed as % of feed unless otherwise stated.
†Isolated soya protein, Ralston Purina Co. Inc., St. Louis, MO, USA.
‡Lactose predigested milk powder (Digestelact, Sharpe Laboratories, Pty. Ltd, Artarmon, NSW.
§The trace mineral mix contained 4·01 g $MnSO_4.H_2O$, 0·548 g $ZnSO_4.7H_2O$, 0·477 g $CuSO_4.5H_2O$, 4·4 mg Na_2SeO_3, 0·79 g KI.
‖The major mineral mix contained 139·3 g NaCl, 389·0 g KH_2PO_4, 57·3 g $MgSO_4$, 381·4 g $CaCO_3$.
¶The vitamin premix contained (in mg/g): menadione, 0·5; choline, 200; *p*-aminobenzoic acid, 10; inositol, 10; niacin, 4; calcium D-pantothenate, 4; riboflavin, 0·8; thiamin HCl, 0·5; pyridoxine HCl, 0·5; folic acid, 0·2; biotin, 0·04; vitamin B_{12}, 0·003; vitamin A, 2000 U; vitamin E, 10 U.
The diets were formulated to contain equal amounts of protein, fat, Ca, P, Fe and vitamin D.

dried whole milk or dried lactose-hydrolysed (LH) milk as outlined in Table 1. All of the diets contained 20% protein and 20% fat and were adjusted to provide equal amounts of Ca, P, Fe and vitamin D. Half of the rats in each dietary group were given 20 μg Pb (as lead acetate)/g diet. The rats were housed in hanging, wire-bottomed, stainless-steel cages. Food and deionized water were provided *ad lib.* and food intake was monitored. After 8 wk, biochemical tests for lead exposure were performed. Blood was removed by heart puncture and then the animals were killed and the bone, tooth, brain, kidney and liver were removed for lead analysis.

Urinary δ-aminolaevulinic acid. Urinary δ-aminolaevulinic acid (ALA) was measured in 24-hr urine samples that had been preserved with 1 ml 1 N-acetic acid. ALA was determined in 1 ml aliquots of urine according to the method of Tomokuni & Ogata (1972).

Free erythrocyte protoporphyrin. A micromethod was used to measure free erythrocyte protoporphyrin (FEP) in heparinized blood samples obtained by heart puncture. A 20-μl aliquot of blood was analysed by the method described by Piomelli (1973). Fluorescence was measured on a Hitachi 650-10M fluorescence spectrometer using an excitation wavelength of 405 nm and an emission wavelength of 610 nm. The fluorometer was standardized using a coproporphyrin I standard (Sigma Chemical Co, St. Louis, MO, USA) containing 0·5 μg coproporphyrin/ml. The haematocrit of the blood sample was measured so that results could be expressed as FEP/100 ml red blood corpuscles.

ALA dehydratase. The activity of ALA dehydratase was measured in heparinized blood samples both with and without the addition of dithiothreitol (DTT). DTT protects protein sulphydryl groups from inactivation by lead and other compounds and therefore enzyme activity measured in the presence of DTT is referred to as 'activated' and in the absence of DTT as 'non-activated'. The percentage inhibition is 1 minus the ratio of non-activated to activated ALA dehydratase activity expressed as a percentage. The method used was essentially that described by Sassa, Granick & Kappas (1975) except that blood sample and reagent volumes were increased tenfold.

Tissue-lead analysis. Accurately weighed tissue samples (approximately 1 g brain, liver and kidney, 0·4 g femur and 0·05 g tooth) were digested with mild heating in low-lead nitric acid (nitric acid for foodstuffs analysis, BDH Chemicals Ltd, Poole, England). Whole blood (300 μl) was treated with Triton X-100 (600 μl) and analysed for lead according to the method of Kubasik, Volosin & Murray (1972). Lead concentration was measured in the prepared blood samples and in appropriately diluted digested tissue samples using a Varian AA-575 atomic absorption spectrophotometer fitted with a CRA-90 carbon

Table 2. *Body weight and food intake of male Wistar rats fed soya, milk or lactose-hydrolysed (LH) milk diets, with or without added lead, for 8 wk (experiment 1)*

Diet	Body weight (g)	Food intake (g)
Soya	240 ± 18	638 ± 28
Soya + Pb	232 ± 18	599 ± 65
Milk	214 ± 15*	620 ± 59
Milk + Pb	219 ± 12†	602 ± 41
LH milk	232 ± 14	640 ± 68
LH milk + Pb	236 ± 16	648 ± 46

*Significantly lower (Student's *t*-test) than the corresponding values for rats fed soya ($P < 0.01$) or LH-milk ($P < 0.02$) without added lead.
†Significantly lower (Student's *t*-test) than the corresponding value for rats fed LH-milk + Pb ($P < 0.02$).
Values are means ± 1SD for groups of ten rats. The amount of lead added to the diets was 20 μg (as lead acetate)/g.

rod atomizer and an ASD-53 automatic sample dispenser.

Statistical analyses. The statistical difference between groups was ascertained using Student's *t*-test.

Experiment 2

Two groups of 10 weanling male Wistar rats (weighing 45–50 g) were fed the soya and milk diets used in experiment 1 in the following feeding regimes: soya group—5 g soya diet containing lead (60 μg/g) in the morning and free access for 2 hr to the soya diet containing no lead in the evening; milk group—5 g soya diet containing lead (60 μg/g) in the morning and free access to the milk diet containing no lead in the evening. After 8 wk, biochemical tests for lead exposure were conducted and tissue-lead concentrations were measured as described for experiment 1.

RESULTS

Experiment 1

Body weight and food intake. The ingestion of 20 μg lead/g feed did not affect the body weight or food intake of rats in any of the dietary groups (Table 2). Rats fed the milk diets with or without added lead had significantly lower body weights compared with those given the soya or LH-milk diets with or without added lead. This slightly lower weight gain of the milk-fed rats cannot be accounted for solely by differences in food intake between the groups. Slightly poorer digestion and utilization of the milk diet due to its high lactose content probably contributed to the lower body weights of the milk-fed rats. Mild diarrhoea occurred in half of the milk-fed rats early in the experiment but this tended to abate with time. None of the soya- or LH milk-fed rats showed any evidence of diarrhoea.

Tissue-lead concentrations. Lead supplementation of the diet increased bone- and tooth-lead concentrations 50 to 100 fold compared with those in rats fed the same diet without lead supplementation. The increase in bone- and tooth-lead was significantly greater in rats fed the milk diet compared to those fed the soya or LH-milk diets (Table 3). This increased lead content represented a real increase in lead in relation to bone calcium since the Pb:Ca ratio was significantly greater in the group fed the lead-supplemented milk diet compared with that of the groups fed the lead-containing soya or LH-milk diets, while the total femur weight and calcium content were similar for all groups (Table 4).

Feeding lead significantly increased kidney, liver and brain lead contents. Rats fed the lead-containing milk diet accumulated significantly more lead in the kidney, liver and brain than those fed lead in either the soya or the LH-milk diet. All of the rats fed the lead-supplemented diets had significantly higher blood lead than the rats fed the same, unsupplemented diet (Table 3). However, the blood-lead content did not reflect differences in lead concentration in other tissues. Lead concentrations in the tissues of lead-exposed rats varied considerably with the femur having the highest levels followed by the tooth, kidney, liver, blood and brain.

Biochemical tests of lead toxicity. Urinary ALA was not influenced by dietary lead; however, with or without supplementary lead, the groups fed LH milk excreted significantly more ALA in the urine than those fed soya or milk diets (Table 5). FEP did not

Table 3. *Lead concentrations in tissues of male Wistar rats fed soya, milk or lactose-hydrolysed (LH) milk diets, with or without added lead, for 8 wk (experiment 1)*

Diet	Concentration of lead in Femur (μg/g)	Tooth (μg/g)	Kidney (ng/g)	Liver (ng/g)	Brain (ng/g)	Blood (μg/100 ml)
Soya	0·36 ± 0·10	0·14 ± 0·07	23 ± 20	48 ± 31	6 ± 8	3·7 ± 1·8§
Soya + Pb	32·72 ± 4·13*	10·46 ± 0·80*	1755 ± 532*	327 ± 73*	42 ± 4*	16·9 ± 5·2*‖
Milk	0·31 ± 0·15	0·13 ± 0·08	30 ± 24	57 ± 16	10 ± 12	10·3 ± 2·8
Milk + Pb	44·78 ± 5·76*†	13·00 ± 1·21*†	2850 ± 335*†	413 ± 63*‡	59 ± 19*‡	30·6 ± 4·1*
LH milk	0·33 ± 0·12	0·15 ± 0·07	30 ± 25	39 ± 24	2 ± 5	9·2 ± 3·1
LH milk + Pb	28·92 ± 3·86*	8·20 ± 1·41*	1859 ± 378*	306 ± 57*	37 ± 7*	30·3 ± 6·3*

*Significantly greater than value for rats given the same diet without lead ($P < 0.001$).
†Significantly greater than values for rats given soya + Pb or LH milk + Pb ($P < 0.001$).
‡Significantly greater than values for rats given soya + Pb ($P < 0.02$) or LH milk + Pb ($P < 0.01$).
§Significantly less than values for rats given milk or LH-milk diets without added lead ($P < 0.001$).
‖Significantly less than values for rats given milk + Pb or LH milk + Pb ($P < 0.001$).
Values are means ± 1SD for groups of 10 rats. The amount of lead added to the diet was 20 μg (as lead acetate)/g. Statistical differences between groups were ascertained using Student's *t*-test.

Table 4. *Fat-free dry weights and calcium contents of femurs of male Wistar rats fed soya, milk or lactose-hydrolysed (LH) milk diets, with or without added lead, for 8 wk (experiment 1)*

Diet	Femur fat-free dry weight (mg)	Calcium content (mg) of femur	Femur Pb:Ca, $\times 10^{-6}$
Soya	475 ± 41	108 ± 17	1·6 ± 0·4
Soya + Pb	452 ± 46	100 ± 15	148·8 ± 21·9*
Milk	457 ± 47	107 ± 15	1·4 ± 0·6
Milk + Pb	473 ± 46	107 ± 14	198·4 ± 30·1*†
LH milk	478 ± 41	109 ± 18	1·6 ± 0·6
LH milk + Pb	473 ± 33	106 ± 10	129·2 ± 20·7*

*Significantly greater (Student's *t*-test) than value for groups given the same diet without added lead ($P < 0.001$).
†Significantly greater (Student's *t*-test) than values for groups given soya + Pb or LH milk + Pb ($P < 0.001$).
Values are means ± 1SD for groups of ten rats. The amount of lead added to the diet was 20 μg (as lead acetate)/g.

increase in rats fed lead-supplemented diets except in the LH-milk group. Among the lead-supplemented rats, those fed LH milk had higher concentrations of FEP than those fed milk or soya, despite the fact that the LH-milk group had similar tissue lead concentrations to the soya group and actually had significantly lower tissue lead levels than the milk group. These results indicate that FEP and urinary ALA do not accurately reflect increases in body lead burden in animals exposed to low concentrations of lead over the period of time tested in these studies.

Experiment 2

Body weight and food intake. Rats consuming the soya + Pb and milk diets consumed significantly less food than the rats given the soya + Pb and soya diets (Table 6). The amount of the soya plus lead diet eaten by both groups during the morning feed was similar, and so both groups were exposed to the same amount of lead. The milk group, however, consumed less diet during the afternoon feed when food was available *ad lib.* This difference in food intake was reflected in the significantly lower body weight of the group fed soya + Pb and milk.

Tissue lead concentrations. There were no differences between the two groups in lead concentrations in any tissue (Table 6). As in experiment 1, significantly more lead accumulated in calcified tissues than in soft tissues. Despite the fact that the lead was presented in high concentration in one meal rather than throughout the day the tissue-lead concentrations were similar in the two experiments (Tables 3 & 6). The femur fat-free weight and calcium content were lower in the rats fed soya + Pb and milk, reflecting the smaller size of these rats; however, the Pb:Ca

Table 5. *Results of biochemical tests on male Wistar rats fed soya, milk or lactose-hydrolysed (LH) milk diets, with or without added lead, for 8 wk (experiment 1)*

Diet	Urinary ALA (μg/24 hr)	FEP (μg/100 ml RBC)	ALA dehydratase (U/litre RBC) non-activated	ALA dehydratase (U/litre RBC) activated	Inhibition (%) of ALA dehydratase
Soya	34 ± 14	70·5 ± 10·4	2·24 ± 0·28‖	6·66 ± 1·49	58·6 ± 8·6
Soya + Pb	29 ± 11	70·2 ± 10·0	0·97 ± 0·40	5·01 ± 1·33	79·3 ± 9·7**
Milk	48 ± 20	65·2 ± 7·1	2·48 ± 0·58‖	6·41 ± 1·51	60·5 ± 8·4
Milk + Pb	38 ± 12	68·7 ± 13·0	1·25 ± 0·46	5·39 ± 1·49	76·3 ± 6·6**
LH milk	67 ± 15*	74·0 ± 7·3‡	2·94 ± 0·39‖¶	6·24 ± 0·99	51·6 ± 10·7
LH milk + Pb	59 ± 20†	86·4 ± 10·5§	1·54 ± 0·34¶	5·45 ± 1·00	71·2 ± 6·2**††

ALA = δ-Aminolaevulinic acid FEP = Free erythrocyte protoporphyrin
RBC = Red blood cells
*Significantly greater than the values for the groups given soya ($P < 0.001$) or milk ($P < 0.05$) without added lead.
†Significantly greater than the values for the groups given soya + Pb ($P < 0.001$) or milk + Pb ($P < 0.01$).
‡Significantly greater than the value for the group given milk without added lead ($P < 0.05$).
§Significantly greater ($P < 0.01$) than the values for groups given soya + Pb, milk + Pb or LH milk.
‖Significantly greater than the non-activated ALA dehydratase values for groups given the corresponding diets with added lead ($P < 0.001$).
¶Values for groups given LH milk or LH milk + Pb are significantly higher than the non-activated ALA dehydratase values for groups given soya or soya + Pb, respectively ($P < 0.01$).
**Significantly greater than the values for groups fed the same diets without added lead ($P < 0.001$).
††Significantly less than the values for the rats given soya + Pb ($P < 0.05$).
Values are means ± 1SD for groups of 10 rats. The amount of lead added to the diets was 20 μg (as lead acetate)/g. Statistical differences between groups were ascertained using Student's *t*-test. Activated and non-activated ALA dehydratase were measured in the presence and absence, respectively, of dithiothreitol.

Table 6. *Body weight, food intake and lead concentrations in the tissues of male Wistar rats fed soya diets containing 60 μg lead/g in the morning and either soya or milk without added lead in the evening for 8 wk (experiment 2)*

Parameter	Values for rats given: Soya + Pb and soya	Soya + Pb and milk
Body weight (g)	219·6 ± 14·4	197·1 ± 16·6**
Food intake (g)	571·6 ± 31·3	508·7 ± 39·1***
Lead intake (mg)	13·57 ± 0·77	13·48 ± 0·37
Lead concn (μg/g tissue) in:		
Femur	25·10 ± 4·56	25·94 ± 3·91
Tooth	11·76 ± 3·12	11·57 ± 3·35
Kidney	1·74 ± 0·37	1·50 ± 0·48
Liver	0·321 ± 0·064	0·305 ± 0·040
Brain	0·042 ± 0·021	0·042 ± 0·019
Blood-lead concn (μg/100 ml)	17 ± 3	15 ± 4
Femur: fat-free dry weight (mg)	369 ± 21	340 ± 32*
calcium content (mg)	81 ± 6	77 ± 8
Pb:Ca ratio × 10^{-6}	115 ± 23	114 ± 20

Values are means ± 1SD for groups of 10 rats, and those marked with asterisks are significantly different (Student's *t*-test) from the corresponding values for rats given soya + Pb and soya (*$P < 0·05$; **$P < 0·01$; ***$P < 0·001$).

ratio in the femur was the same for both groups (Table 6).

Biochemical tests of lead toxicity. Urinary ALA excretion was similar in both groups (Table 7). While FEP/100 ml red blood cells was greater in the soya + Pb and milk group, the activity of erythrocyte ALA dehydratase actually showed significantly greater inhibition in the soya + Pb and soya group (Table 7). Since both groups had similar concentrations of lead in all of the tissues it is clear that these biochemical tests are not reliable indications of body lead burden in animals subjected to chronic low levels of lead exposure.

DISCUSSION

The present experiments indicate that milk increased the absorption and retention of orally ingested lead when milk and lead were fed together but not when milk and lead were fed at separate meals. In experiment 1, rats fed the lead-containing milk diet accumulated significantly more lead in all body tissues than the groups fed the lead-supplemented soya or LH-milk diets (Table 3). This observation on the long-term influence of milk on lead metabolism is in agreement with short-term studies on the influence of milk on the uptake of lead from the intestine. Kello & Kostial (1973) demonstrated that a single dose of radioactive lead was absorbed more rapidly in rats consuming milk than in animals given a milk-free diet. Although the influence of milk on lead absorption is greater in suckling rats than in older rats, Kostial, Kello, Jugo, Rabar & Maljković (1978) found a substantial increase in ^{203}Pb absorption in mature rats fed milk diets. Stephens & Waldron (1975) concluded from their review of the literature that there was strong evidence that whole cows' milk increased lead absorption, and suggested that

Table 7. *Results of biochemical tests on male Wistar rats fed soya diets containing 60 μg lead/g in the morning and either soya or milk without added lead in the evening for 8 wk (experiment 2)*

Parameter	Values for rats given: Soya + Pb and soya	Soya + Pb and milk
Urinary ALA (μg/24 hr)	46 ± 17	41 ± 16
FEP (μg/100 ml RBC)	41·2 ± 4·7	48·2 ± 7·1*
ALA dehydratase:		
Non-activated (U/100 ml RBC)	2·37 ± 0·84	3·92 ± 1·03*
Activated (U/100 ml RBC)	12·18 ± 2·15	12·86 ± 2·27
Percentage inhibition	78·0 ± 2·15	69·5 ± 5·9**

ALA = δ-Aminolaevulinic acid FEP = Free erythrocyte protoporphyrin
RBC = Red blood cells

Values are means ± 1SD for groups of 10 rats, and those marked with asterisks are significantly different (Student's *t*-test) from the corresponding values for rats given soya + Pb and soya (*$P < 0·02$; **$P < 0·01$). Activated ALA dehydratase was measured in the presence of dithiothreitol and non-activated ALA dehydratase was determined in the absence of dithiothreitol.

milk should no longer be regarded as a prophylactic agent against lead poisoning.

The reason why milk increases lead content cannot be explained by previous studies on the interactions of lead with minerals that are present in milk. Shields & Mitchell (1941), using low levels of dietary lead (15–33 μg/g), found that diets low in calcium and phosphorus increased tissue-lead concentrations in rats. Supplementation of normal rat diets with calcium phosphate significantly decreased the absorption of an orally administered tracer dose of ^{203}Pb (Meredith, Moore & Goldberg, 1977). Since the retention of lead varies inversely with dietary calcium content (Moore, 1979), it would appear that adequate intake of calcium and phosphorus are protective against lead absorption and retention. Milk is a rich source of both calcium and phosphorus, and yet it appears to increase the body burden of lead rather than to protect against lead toxicity in chronically exposed animals.

Iron-deficient animals absorb greater amounts of lead than those whose diets are adequate with respect to iron (Barton, Conrad, Nuby & Harrison, 1978). Since milk is low in iron as well as other trace minerals, Kostial, Rabar, Bladuša & Šimonović (1980) investigated the greater rate of absorption of heavy metals in rats fed a milk diet and found that it was not due to the low iron content of the diet. Likewise, differences in iron status cannot explain the greater retention of lead in the milk-fed rats in the present study since all of the diets contained the same amount of iron (70 μg/g). In experiment 1, rats fed LH milk plus lead or soya plus lead had similar lead concentrations which were substantially lower than those of rats fed lead-supplemented milk. The LH-milk diet had the same composition as the milk diet except that the lactose had been enzymatically hydrolysed to glucose and galactose. This strongly suggests that lactose is the factor in milk responsible for increasing lead absorption and tissue-lead concentrations. Although it has long been known that lactose increases the absorption of calcium from the small intestine of many mammals, the mechanism of this action has not been fully explained. Armbrecht & Wasserman (1976) monitored uptake of ^{45}Ca from isolated everted gut sac preparations and concluded that lactose does not complex with calcium itself but rather that it interacts with the absorptive cells of the small intestine and increases their permeability to calcium. It is possible that lactose could increase the permeability of mucosal cells to lead and other divalent cations as well as to calcium. Indeed, lactose has been shown to enhance the absorption of radioactive strontium in the rat (Lengemann, Wasserman & Comar, 1959). Lactose increases the passive absorption of calcium and has the greatest effect in the ileum (Armbrecht & Wasserman, 1976). In order for lactose to increase calcium absorption it must be present in the same intestinal segment as calcium (Lengemann *et al.* 1959). The results of experiment 2 also demonstrate that milk increases the body lead burden only when milk and lead are consumed at the same time.

Although consumption of lactose-containing milk increases lead absorption and tissue-lead concentrations, this should not be interpreted to mean that the consumption of dairy products ought to be limited. Dairy products provide the major source of dietary calcium, contributing approximately 75% of calcium present in a normal mixed diet (Australian Bureau of Statistics, 1979) and most of the dairy products eaten still contain some lactose. The removal of lactose-containing foods would adversely affect calcium status in most people. Since diets low in calcium also enhance lead absorption, reduction of intake of dairy foods would not necessarily be protective against orally ingested lead. The better long-term approach to the problem would be to minimize the quantity of lead ingested from all sources.

The relatively low levels of dietary lead given over a long period of time in these experiments makes the present system a good model for the chronic lead exposure experienced by most populations in industrialized societies. The tissue distributions of lead in these experiments reflect the organism's handling of lead under physiological conditions rather than the responses to acute lead toxicity that have been observed in many experiments in which high concentrations of dietary lead (500–2000 μg/g) have been used.

Lead levels in the environment have increased substantially since prehistoric times. Present day diets contain 100 times more lead than is estimated to have been in diets of prehistoric peoples. Atmospheric lead has increased from an estimated 0·04 ng/m^3 to 800 ng/m^3 while lead in water has increased from 0·02 to 15 μg/litre (Settle & Patterson, 1980). Approximately 70% of the lead man encounters in non-industrial situations is derived from food (Settle & Patterson, 1980). Because of this increasing presence of lead in the environment, it is important to be able to monitor any health effects of chronic exposure to low levels of lead. Current methods for assessing the health hazards associated with lead exposure rely on assays of lead concentrations in body fluids and of the activity of enzymes affected by lead, and on the determination of the presence of elevated levels of various metabolites. The measurement of lead in whole blood has been widely used to monitor for potential lead hazards. Blood-lead levels, however, are a better measure of recent lead exposure than of total body lead burden (Chisholm, Barrett & Mellits, 1975; Department of Health and Social Security, 1980; Gross, Tsay & Middendorf, 1976; World Health Organization, 1980), and, therefore, may not be a sensitive indicator of potential health hazards for the general public. The level in children that represents cause for concern has been set by the National Health and Medical Research Council Public Health Advisory Committee (1979) at 30 μg/100 ml. In both of the experiments described in this paper the blood-lead levels of rats exposed to 20 μg lead/g dry diet were not greater than 30 μg/100 ml. Despite the fact that blood-lead levels in these experiments did not exceed current standards set for humans, the animals did accumulate high concentrations of lead in both calcified and soft tissues.

It is now fairly well established that lead can affect several steps in the synthesis of haem. ALA dehydratase, which catalyses the formation of porphobilinogen from δ-aminolaevulinic acid, and haem synthetase, which incorporates iron into protoporphyrin IX, are particularly affected by lead (Chisholm, 1971). In-

hibition of these enzymes by lead results in increased concentrations of various intermediate compounds. Free protoporphyrins accumulate in the erythrocytes and higher levels of δ-aminolaevulinic acid are excreted in the urine.

An increase in urinary ALA is one of the most specific indicators of lead toxicity since the only other conditions that have been shown to elevate ALA levels in the urine are acute alcoholic intoxication, and the genetic condition, acute intermittent porphyria (Posner, Damstra & Nriagu, 1978). Despite the fact that urinary ALA is a specific indicator for lead toxicity it apparently lacks sensitivity. Urinary ALA appears to be a more useful test for industrial workers exposed to high levels of lead than for children exposed to low levels of lead, where high rates of false positive and false negative results are recorded (Posner *et al.* 1978). The apparent lack of sensitivity of urinary ALA is also demonstrated in the present studies since feeding 20 μg lead/g did not cause an increase in urinary ALA in the rats after 8 wk.

Porphyrins accumulate in erythrocytes following lead exposure, and since they are retained within the cell throughout its life span, FEP concentrations represent a fairly long-term and relatively stable response to lead exposure. Alessio, Bertazzi, Monelli & Toffoletto (1976) have found that in workers previously exposed to lead, FEP is more closely correlated with chelatable lead (lead excreted following the administration of ethylenediaminetetraacetic acid, disodium salt) than with blood-lead levels. These researchers concluded that FEP concentrations were a good indication of internal, mobilizable lead in previously exposed workers. The rats given 20 μg lead/g in the present experiments did not have elevated levels of FEP and all values were well below 160 μg/100 ml, the value for a positive test for humans suggested by Piomelli (1977). It is possible that FEP would have increased in these rats if the exposure period was extended beyond 8 wk since the FEP effect is slower to develop than are changes in blood-lead levels or ALA dehydratase.

Erythrocyte ALA dehydratase activity is very sensitive to lead, and the enzyme shows inhibition at blood-lead levels as low as 10–15 μg/100 ml (Zielhuis, 1975). Rats in experiment 1 given 20 μg lead/g in the diet showed greater ALA dehydratase inhibition than those without added lead. However, the group given lead in milk did not have greater ALA dehydratase inhibition than did the other groups given lead in soya or LH-milk diets, despite the fact that the milk plus lead group had 30–60% more lead in the body tissue than did the other two lead-treated groups. This suggests that erythrocyte ALA dehydratase inhibition may reflect current blood-lead levels but may not be a good indicator of total body lead burden.

These studies indicate the need for further research on the interaction between dietary factors and lead toxicity and also the need for more sensitive methods for the assessment of adverse health effects resulting from chronic exposure to low levels of lead.

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PESTICIDE RESIDUES IN HUMAN MILK

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Abstract—A total of 154 samples of human milk collected 3–6 days after parturition were obtained from four hospitals in Quebec. Samples were analysed by electron-capture gas–liquid chromatography for total DDT (DDT + DDE), aldrin, γ-HCH (lindane) and polychlorinated biphenyls (PCBs). In 93% of the milk samples the PCB level exceeded that equivalent to the Acceptable Daily Intake proposed by the USA for PCBs, the mean PCB level (± SD) for the 154 samples being 0·837 ± 0·529 mg/kg milk fat. The Codex Alimentarius Commission maximum residue limits were exceeded in 30% of the samples with regard to total DDT (mean ± SD 1·087 ± 0·880 mg/kg milk fat) and in 3% with regard to γ-HCH (0·047 ± 0·131 mg/kg) and aldrin (0·041 ± 0·068 mg/kg). The age of the donors showed a positive correlation with PCB concentrations, while previous breast-feeding was inversely correlated with γ-HCH levels. A significant positive correlation was observed between cigarette smoking and the DDE content of human milk fat. Also of importance were the higher levels of PCB associated with residence in an industrialized area.

INTRODUCTION

Breast feeding offers many advantages to infants and mothers, including improved nutrition, increased resistance to infection, protection against allergy and better mother–child relationships. Lately, there has been concern about the levels of pesticide residues and other contaminants in human milk, and Table 1 summarizes concentrations of total DDT, γ-HCH (lindane) and polychlorinated biphenyls (PCBs) that have been found in human milk in various parts of the world. These data indicate that human milk generally contains DDT in concentrations exceeding the practical residue limit for cows' milk of 1·25 mg/kg on a fat basis (about 0·05 mg/kg whole milk) proposed by the Joint FAO/WHO Experts on Pesticide Residues (1973) on the recommendation of the Codex Alimentarius Commission (Joint FAO/WHO Food Standards Programme, 1974). However, since many countries restricted the use of DDT in the late 1960s, the levels of DDT and its metabolites in human milk have consistently decreased. This was demonstrated by three studies in Canada (Table 1), where levels of DDT decreased from 0·139 mg/kg in 1969 to 0·044 mg/kg in 1975–1976 (Health Protection Branch, Health and Welfare Canada, Information Letter 31 March 1978).

More recently, concern has been focused on the presence of PCBs in human milk. Current toxicological data are scarce and all the surveys recorded in Table 1 show PCB levels of human milk above the tolerance dose of 1 μg/kg body weight proposed by the United States (Health Protection Branch Information Letter 31 March 1978), since if an infant drinks approximately 150 ml milk/kg body weight, the milk must contain less than 10 μg PCBs/kg whole milk if this proposed limit is not to be exceeded. However, the use of PCBs was also restricted several years ago and levels in human milk will probably decrease as have those of DDT.

Further studies of organochlorine residues have now been conducted on samples of human milk collected at several hospitals in the province of Quebec.

EXPERIMENTAL

Sampling. The 154 human-milk samples studied were obtained randomly between October 1978 and January 1979 from four hospitals in the province of Quebec. Three of the hospitals, the Sainte-Justine Hospital in Montreal (32 samples), Charles Le Moyne Hospital in Longueuil (10) and Christ-Roi Hospital in Quebec City (70), received mothers residing mainly in city areas. The remaining 42 samples were collected in Levis, which is located in a more rural area.

The milk samples were collected 3–6 days after parturition. All were manually expressed after nursing, placed in chemically clean glass bottles and stored at −10°C until assayed. Three milk samples, usually collected after the feeds at 09.00, 12.00 and 17.00 hr, were provided by 87% (130) of the women, while 3% (4) and 10% (16) of the subjects donated one and two samples, respectively. Donors were questioned about smoking habits, weight gain or loss, parity, exposure to pesticides, occupational history and residence since birth.

Analyses

Materials. All glassware was tested for possible impurities. The solvents used were glass-distilled and each batch was tested by gas chromatography after concentration from 100 to 0·5 ml. Florisil adsorbent (60–100 mesh; Fisher Scientific Co., Pittsburgh, PA, USA) was treated at 550°C and maintained at 125°C prior to use. Silica gel (Fisher grade 950, 60–200 mesh; Fisher Scientific Co.) was also kept at 125°C. Anhydrous sodium sulphate was treated at 800°C and kept at 125°C.

Table 1. *Organochlorine residue levels in human milk in various countries*

Location	Yr of study	No. of samples	Mean residue content (mg/kg human milk) Total DDT*	PCBs	γ-HCH	Reference†
UK	1965	19	0·128			
Sweden	1970	22	0·107	0·015		
Belgium	1969	20	0·119			
Rumania	1969	100	0·530			
Germany	1970	43	0·112	0·103		
Russia	1970	366	0·230			
Netherlands	1971	50	0·048			
Australia	1973	45	0·064			Syali (1973), Miller & Fox (1973)
Iran	1974/76	131	0·024			Hashemy-Tonkabony & Fakeminassab (1977)
Norway		50‡	0·082		0·010	Bakken & Seip (1976)
Finland	1973/74	49	0·058	0·024		Vuori, Tyllinen, Kuitunen & Paganus (1977)
Georgia		5	0·070			Curley & Kimbrough (1969)
Pennsylvania	1970	53	0·090		0·003	Kroger (1972)
Seven US cities	1970/71	138	0·17			Wilson, Locker, Ritzen, Watson & Schoffner (1973)
Colorado		40§	0·030–0·500	0·40–0·1		Savage, Tessari, Malberg, Wheeler & Bagby (1973)
Arizona		6	0·100–0·500			Hagyard, Brown, Stull, Whiting & Kemberling (1973)
Texas		28	0·108		0·007	Dyment, Hebertson, Gomes, Wiseman & Hornabrook (1971)
Mississippi/Arkansas	1974	38	0·447			Woodard, Ferguson & Wilson (1976)
Nashville, TN	1974	14	0·075			Woodard *et al.* (1976)
Missouri	1973	51	0·022		0·004	Jonsson, Liu, Armbruster, Kettelhut & Drucker (1977)
Quebec	1967/68	29	0·131		0·075	Ritcey, Savary & McCully (1972)
Ontario	1967/68	25	0·169		0·071	Ritcey *et al.* (1972)
Nova Scotia	1972	9	0·025	0·018		Musial, Hutzinger, Zitko & Crocker (1974)
New Brunswick	1972	6	0·048	0·022		Musial *et al.* (1974)
Canada	1975/76	100	0·044	0·012		Mes & Davies (1979), Health Protection Branch (HWC). Information Letter 31 March 1978
			0·078			
	1969		0·139			

*The sum of *p,p'*-DDE, *p,p'*-DDT and related compounds, if reported.
†Where no reference is given, the data are taken from those cited by Ritcey *et al.* (1972).
‡Only 17 subjects for γ-HCH analyses.
§Only eight subjects for PCB analyses.

Table 2. *Distribution of PCB and chlorinated pesticides in 154 samples of human milk collected in the province of Quebec in 1978–1979*

Compound	Residue level (mg/kg milk fat) Mean	SD	Mode	Median	Range	Residue level (mg/kg whole milk)* Mean	SD
PCB†	0·837	0·529	0·517	0·742	0–4·34	0·029	0·019
γ-HCH	0·047	0·131	0·019	0·025	0–1·56	0·002	0·0001
Aldrin	0·041	0·068	0·011	0·026	0–0·74	0·001	0·003
p,p'-DDE	0·883	0·773	0·314	0·699	0·01–6·79	0·032	0·028
p,p'-DDT	0·204	0·262	0·141	0·145	0–2·51	0·007	0·009
Total DDT	1·087	0·880	0·835	0·856	0·01–6·81	0·039	0·031

*The fat content of the milk was not determined; these figures were calculated, assuming 3·5% as the average fat content of human milk up to 10 days *postpartum*, so that the data could be compared with those of other studies for which results were given only on a whole-milk basis.
†One human milk sample containing 29·017 mg PCB/kg is not included in the results.

Extraction, clean-up and separation of PCBs from pesticides

The partition method of McLeod & Ritcey (1973) was used to extract the residues of pesticides from milk. Each extraction required at least 0·1 g fats and was made in duplicate. The clean-up of the extract was based on the method of Chau (1972) except that the adsorbent-column dimensions were modified according to Storherr, Murray, Klein & Rosenberg (1967). These columns (9 cm × 1 cm ID) were fitted with Florisil adsorbent covered with a layer of sodium sulphate and were eluted with 50 ml benzene and then 150 ml hexane for washing and conditioning. Samples were evaporated to 1 ml and deposited on to the top surface of the columns. PCBs and some pesticides were eluted with 50 ml hexane percolating at a rate of 3–4 ml/min. The remaining pesticides were eluted with 50 ml benzene–hexane (5:1, v/v).

Columns of silica gel were used for separating PCBs from pesticides, as described by Snyder & Reinert (1971). Dimensions, washing and conditioning were the same as for the columns described above. For optimum separation of PCBs from pesticides in the hexane fraction previously obtained, these columns were eluted first with 50 ml pentane and then with 50 ml benzene.

Levels of recovery for the complete extraction and separation procedure were 66% for DDT, 100% for TDE, 88% for DDE, 64% for γ-HCH, 95% for aldrin and 59% for PCBs.

Chromatography. All samples were chromatographed on a Microtek MT-220 gas–liquid chromatograph using a ^{63}Ni (10 mCi) electron-capture detector, modified as described by Gosselin, Martin & Boudreau (1974) but utilizing a constant-current pulse mode, and a glass column (4 mm ID) packed with 4% SE-30 + 6% QF-1 on Gas-Chrom Q (80–100 mesh). The chromatograph was operated at an oven temperature of 185°C, an inlet temperature of 225°C, a detector temperature of 350°C, a carrier-gas flow of 50 ml N_2/min and a detector scavenger-gas flow of 20 ml N_2/min, and used a constant-current pulse mode electrometer at an attenuation of 8 and at a relative pulse width of 4 μsec.

Quantitative evaluation. PCBs were estimated by measuring the sum of the heights of peaks and comparing that figure with that of the standard, Aroclor 1254. For pesticides, the heights of peaks were compared with the peak height for a known quantity of pure standard.

RESULTS AND DISCUSSION

The mean age of the donors was 26·8 ± 3·8 yr (18–38 yr). Over half of the mothers (62%) were nursing their first infant, 28% their second, 7% their third and 3% their fourth.

PCBs were present in 99% of the samples, while γ-HCH was found in 95%, aldrin in 98% and *p,p'*-DDT in 99%. All samples contained *p,p'*-DDE, but none contained *p,p'*-TDE at detectable levels. Table 2 lists the concentrations of DDE, total DDT, PCB, aldrin and γ-HCH found in the samples. The highest PCB concentration was in the milk of a 29-yr-old woman who was nursing her first child and was in her third day of lactation. She had lived in an industrialized area of Quebec City for 5 yr, very close to an incinerator. The average total DDT (0·039 mg/kg) content of the 154 human milk samples analysed in this study was similar to the Canadian average of 0·044 mg/kg found in 1975 by the Health Protection Branch (Information Letter 31 March 1978) but was below the average of 0·131 mg/kg reported by Ritcey *et al.* (1972) in 29 samples collected in 1967–1968 from the province of Quebec.

To date there have been no reported cases of infants suffering damage from these insecticides in milk, but Fahim, Bennett & Hall (1970) reported an increase in deaths among rats suckled by mothers fed large quantities of DDT. On the other hand, Hayes, Dale & Pirkle (1971) reported that adult men fed DDT for 21·5 months in oral doses 550 times higher than the average daily intake showed no definite clinical or laboratory signs of effect during a 5-yr follow-up.

The mean γ-HCH concentration (0·047 mg/kg on a fat basis) found in our study was also lower than the average of 0·075 mg/kg reported by Ritcey *et al.* (1972) for the province of Quebec.

Comparison with tolerance doses

The Codex Alimentarius Commission (Joint FAO/WHO Food Standards Programme, 1974) has set practical residue limits of 1·25, 0·2 and 0·15 mg/kg on a fat basis for total DDT, γ-HCH and aldrin, respectively, in milk. As indicated in Table 3, 30% of the samples exceeded this limit for total DDT, and the limits for γ-HCH and for aldrin were exceeded in 3%. Compared with earlier Canadian studies of insecticides in human breast milk (Health Protection Branch Information Letter 31 March 1978; Musial *et al.* 1974; Ritcey *et al.* 1972), our results show lower overall levels of DDT and γ-HCH.

The US acceptable daily intake (ADI) for PCBs is set at 0·001 mg/kg body weight for newborns (Health Protection Branch Information Letter 31 March 1978).

Table 3. *Comparison between organochlorine residues found in human milk and specified tolerances*

Compound	Tolerance (mg/kg*)	Subjects with milk exceeding residue tolerance: No.	Subjects with milk exceeding residue tolerance: Percentage of total
PCB†	0·01	143	93
γ-HCH	0·2	4	3
Aldrin	0·15	4	3
Total DDT‡	1·25	46	30

*The tolerance for PCBs is presented on a whole-milk basis (derived from the US acceptable daily intake); those for γ-HCH, aldrin and total DDT are Codex Alimentarius Commission maximum residue limits and are given in terms of weight of milk fat.

†One human milk containing 29·017 mg/kg was not included in the calculations.

‡The sum of *p,p'*-DDE and *p,p'*-DDT.

As already mentioned, if an infant drinks approximately 150 ml milk/kg body weight, the milk must contain less than 0·01 mg PCB/kg if this limit is not to be exceeded. As shown in Table 3, 93% of the samples exceeded the US limit for PCBs. Although actions have been undertaken by the Canadian government to reduce human exposure to PCBs, the mean concentration of PCBs in whole milk in this study (0·029 mg/kg) was about 2·5 times higher than the average of 0·012 mg/kg reported in the 1975–1976 survey of 100 samples from across Canada (Health Protection Branch, Information Letter 31 March 1978).

We could not find any Canadian studies on aldrin.

On the basis of available animal toxicity data, the Health Protection Branch of Health and Welfare Canada advised physicians in its Information Letter dated 31 March 1978 to pay particular attention to the mother's history and child's birth weight and development when PCB levels exceeded 50 μg/kg whole milk. In the 1975–1976 Health Protection Branch survey reported in that Information Letter, 98% of the breast milk samples analysed had levels below 50 μg/kg whole milk compared with about 88% below that level in the present study.

Correlation of residue levels with physiological and environmental factors

Age. The results presented in Table 4 indicate that there was a positive correlation between the age of all subjects and the PCB concentration in the milk. For over 40 yr, PCBs were used widely in products such as paints, printers' inks and electrical transformers, and it was only in 1972 that their general use was restricted to certain electrical equipment under carefully controlled conditions. This may explain why the milk from the older nursing mothers contained more PCBs than that from young mothers.

The correlation between the age of the donors and the organochlorine residues varied greatly from one hospital to another. There was a positive correlation between the age of the 42 subjects sampled in Levis and the concentrations of DDE, total DDT and γ-HCH, whereas there was no correlation between PCB concentration and age in these subjects. In the 70 subjects sampled in Christ-Roi Hospital in Quebec City, there appeared to be a positive correlation between the age of the donors and the concentration of PCB, DDE, DDT and total DDT, but there was no correlation between the age of the 42 donors at Sainte-Justine and Charles Le Moyne Hospitals in Montreal and any of the organochlorine residues analysed (Table 4). Some explanation for the differences found in these three hospitals may lie in the type of population they serve. Sainte-Justine Hospital, a children's hospital with a high-risk pregnancy unit, receives subjects from different parts of the city of Montreal with varying degrees of exposure to organochlorine residues. The same situation applies to Christ-Roi Hospital in Quebec City, which offers Le Boyer's natural childbirth method. As for the hospital in Levis, most of the subjects come from the immediate area, which is not as industrialized as other regions involved in this study, and therefore the lack of correlation between age and PCB concentration was to be expected.

Previous breast-feeding. Also of importance was a diminishing γ-HCH concentration with increasing number of children nursed by the mother (correlation coefficient, −0·1260, $P < 0·05$) and with the number of weeks of the nursing periods (correlation coefficient, −0·1286, $P < 0·05$). An absence of correlation between lactation and the other organochlorine residues analysed may be due to the fact that after nursing, mothers were re-exposed to all organochlorine compounds except γ-HCH. Kroger (1972) found that the concentration of DDT in the milk of mothers who had nursed three or more babies was below average.

Cigarette smoking. A significant correlation (correlation coefficient, 0·1159, $P < 0·05$) was observed between cigarette smoking (number of years) and the concentration of DDE in the milk of 111 subjects sampled in Levis and Quebec City. Vuori *et al.* (1977) found a significant correlation between cigarette smoking and the concentration of DDE and total DDT, and cited Bradt & Herrenkohl (1976) as having shown that the mean total DDT in milk was lower for non-smokers than for cigarette smokers, 15% of the variation in total DDT being

Table 4. *Comparison between the age of the subjects and the organochlorine residue concentrations in their milk*

Source of samples	Correlation† of age milk residue levels in milk (determined on a fat basis)					
	PCB	γ-HCH	Aldrin	*p,p'*-DDE	*p,p'*-DDT	Total DDT
All hospitals	0·1032	0·0575	−0·0010	0·1495	0·1109	0·1504
	(154)	(151)	(150)	(150)	(150)	(150)
	0·033*	NS	NS	NS	NS	NS
Christ-Roi	0·1416	0·0876	0·0443	0·1800	0·1471	0·1782
	(70)	(69)	(69)	(69)	(69)	(69)
	0·046*	NS	NS	0·017*	0·042*	0·018*
Levis	0·1086	0·1873	0·0992	0·2544	0·1535	0·2496
	(42)	(42)	(42)	(42)	(42)	(42)
	NS	0·047*	NS	0·011*	NS	0·012*
Ste-Justine and Charles Le Moyne	0·0158	−0·0758	−0·1655	−0·0468	0·0583	−0·0298
	(42)	(40)	(39)	(39)	(39)	(39)
	NS	NS	NS	NS	NS	NS

NS = Not significant ($P \geq 0·05$)

†Each block of figures presents, in descending order, the Kendall correlation coefficient, the number of donors (in parenthesis) and the degree of significance (marked with an asterisk where $P < 0·05$).

Table 5. *Comparison between organochlorine residues in milk and place of residence of donors*

Residue	Mean residue levels (mg/kg fat ± SD) in milk of donors from Rural areas*	Urban areas*	Significance (*P*)
PCB	0·59 ± 0·32	0·86 ± 0·40	0·012
γ-HCH	0·04 ± 0·07	0·03 ± 0·03	NS
Aldrin	0·03 ± 0·03	0·05 ± 0·10	NS
p,p'-DDE	0·58 ± 0·35	0·99 ± 0·99	0·014
p,p'-DDT	0·14 ± 0·11	0·18 ± 0·12	NS
Total DDT	0·72 ± 0·43	1·17 ± 1·02	0·013

NS = Not significant ($P \geq 0.05$)

*This comparison was limited to donors living exclusively in either a rural (19 donors) or an urban (51 donors) locality.

attributable to the number of cigarettes smoked daily. These correlations are understandable when one considers that tobacco plants are regularly sprayed with DDT (Miller & Fox, 1973).

Parity and other maternal factors. No significant correlations were observed between the concentrations of organochlorine residues and the parity of the donors. This may have been because the majority of the mothers (62%) were nursing their first infant. These results are in agreement with those reported in Finland by Vuori *et al.* (1977) for a group of 49 mothers. There appeared to be no correlation between the concentrations of organochlorine residues and the weight gain of the donors or the birth weight of their babies.

Home use of insecticides. The use of aerosol insecticides in the home may lead to respiratory or dermal exposure. In our study, a small percentage (13%) of donors reported the use of aerosol insecticides in the home, but this was not found to be associated with any increased pesticide content in the milk. Similar results were obtained by Wilson *et al.* (1973) in 138 samples of human milk from seven US cities.

Locality of residence. Table 5 indicates that the mean PCB concentration for donors who lived all their life in urban areas was significantly higher than that for donors who resided exclusively in rural areas. (Subjects who lived in both rural and urban areas were not included in this correlation test.) These results were to be expected, as industrial exposure constitutes one of the main sources of contamination with PCBs. Consequently people living in heavily industrialized cities like Montreal and Longueuil and, to a lesser extent, in Quebec City were likely to be exposed to higher concentrations of PCBs than subjects living in the rural area of Levis.

Contrary to our expectations, however, urban mothers also had higher levels of DDE and total DDT than had rural donors. These findings may have been due to the fact that the rural donors, who did not declare any use of insecticides, were probably less exposed to these substances than their urban counterparts because they consumed more fruits and vegetables that were grown in their gardens and were not sprayed with insecticides.

Conclusions

In this study, 93% of the milk samples were found to exceed the level giving the acceptable daily intake proposed by the USA for PCBs, while 30% exceeded the Codex Alimentarius Commission maximum residue limit for total DDT and 3% exceeded those for aldrin and γ-HCH. Compared with earlier Canadian studies, our results show a lower overall level of DDT and γ-HCH and a much higher level of PCBs. Also of importance were the marked geographical differences found in the levels of organochlorine residues in human milk samples; in particular, the PCB concentrations increased with the level of industrialization. The age of the donors showed a positive correlation with PCB concentration, while previous breast-feeding was inversely correlated with γ-HCH levels. A marked positive correlation between cigarette smoking and organochlorine concentrations in human milk provides a further reason for advising mothers not to smoke.

In the light of currently available information, the benefits of breast-feeding outweigh any potential hazards from contaminants. In the literature, we found no reported cases of breastfed infants being harmed by organochlorine residues in human milk. Nevertheless, we feel that organochlorine residues in human milk should be investigated further and that programmes should be expanded to determine whether adverse effects may occur in infants exposed to milk containing organochlorine compounds.

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THE TOXICITY OF ALFALFA SAPONINS IN RATS

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Abstract—Saponins were isolated from aerial alfalfa plants (alfalfa top saponins; ATS) and incorporated into semi-purified diets. Three groups of eight Sprague–Dawley male rats were fed, respectively, a semi-purified control diet or the same diet containing 1 or 2% ATS. The former ATS diet was fed for 6 months, the latter for 2 months. During the first 4 days, rats on ATS showed a depression of food intake, and 1% cholesterol was subsequently added to all the diets. ATS ingestion had no effect on food intake (after day 5), growth or survival, blood urea nitrogen, serum levels of glucose, creatinine, sodium, potassium, chloride, CO_2, uric acid, calcium, phosphorus, total protein, albumin, total and direct bilirubin, alkaline phosphatase or glutamic–oxalacetic transaminase, erythrocyte and leucocyte counts, haemoglobin levels, haematocrit determinations or erythrocyte osmotic fragility. The levels of serum cholesterol and triglycerides were reduced in animals fed 1% ATS for 6 months. Post-mortem examination revealed no differences in gross or microscopic findings among the groups. Thus this study provided no evidence of toxicity associated with ATS in cholesterol-fed rats.

INTRODUCTION

Over 60 million tons of alfalfa hay are fed, presumably without ill effects, to farm animals annually in the United States (Statistical Abstracts of the United States, 1978). However, there are marked species differences in tolerance to the plant. Thus, alfalfa meal incorporated into food at the 10% (w/w) level depresses the growth of chicks (Cooney, Butts & Bacon, 1948) and pigs (Hanson, Becker, Terrill, Jensen & Norton, 1956), whereas rabbits and monkeys grow satisfactorily on diets containing 50–60% alfalfa (Cheeke & Amberg, 1972; Malinow, McLaughlin, Naito, Lewis & McNulty, 1978). Peterson (1950) found that extracts of alfalfa added to food inhibited the growth of chicks and suggested that the material involved was a saponin.

Subsequently, it was shown that food containing 0·1–0·5% alfalfa saponin inhibited the growth of chickens (Anderson, 1957; Heywang & Bird, 1954; Heywang, Thompson & Kemmerer, 1959; Ishaaya, Birk, Bondi & Tencer, 1969) and that this inhibition—in a way similar to that of alfalfa or alfalfa extracts (Peterson, 1950)—was partly prevented by the addition of dietary cholesterol (Anderson, 1957; Ishaaya *et al.* 1969). Moreover, low-saponin alfalfa cultivars are better tolerated than high-saponin cultivars (Cheeke, Kinzell & Pedersen, 1977; Pedersen, Anderson, Street, Wang & Baker, 1972).

The depression of growth was partially prevented by the addition of cholesterol to the diet when alfalfa saponins were fed at the 0·5% level to rats (Wilcox & Galloway, 1961), at the 0·5–1·0% level to mice, and at the 0·5% level to quail (Reshef, Gestetner, Birk & Bondi, 1976). These experiments were conducted for short periods (2–3 wk) and no other toxic effects were reported. However, distention of the rumen was observed in eight of ten ruminants given single 15–75-g doses of alfalfa saponins by stomach tube (Lindahl, Cook, Davis & Maclay, 1954).

We describe here subchronic studies on rats fed alfalfa saponins at the 1 and 2% dietary levels. Possible toxicity was ascertained from records of food intake and body growth, from a variety of blood determinations and from gross and histological study of several organs.

EXPERIMENTAL

Test material. Alfalfa top saponins (ATS) were obtained from alfalfa aerial plants. They were prepared by a modified version of the method of Walter, Van Atta, Thompson & Maclay (1954). The ability to depress intestinal cholesterol absorption was enhanced by partial acid hydrolysis (Malinow, McLaughlin, Papworth, Stafford, Kohler, Livingston & Cheeke, 1977).

Animals and diet. Male Sprague–Dawley rats (body weight c. 170 g) were assigned randomly to three groups each of eight animals. These groups were maintained, respectively, on a semi-purified diet, or the same diet containing 1 or 2% ATS. The semi-purified diet consisted of casein (26%), sugar (46%), Alphacel (19·5%), safflower oil (2%), Hegsted IV salt mix (ICN Nutritional Biochemicals, Cleveland, OH; 4%), vitamins OWP (ICN Nutritional Biochemicals; 2%), crystalline vitamin D_3 (10 μg/100 g) and DL-methionine (0·5%). After the first 4 days, 1% cholesterol was added to all the diets.

Experimental design and conduct. Food intake was measured on 2 or 3 days/wk for the first 8 wk. Body

weights were recorded weekly throughout the experiment. All animals from the 2% ATS group and four randomly selected control rats were killed after 2 months. The remaining animals were killed after treatment for a total of 6 months.

Before being killed, each animal was anaesthetized with ether and a 5-ml blood sample was obtained from the heart for the following determinations: glucose, blood urea nitrogen (BUN), creatinine, sodium, potassium, chloride, CO_2, uric acid, calcium, phosphorus, total protein, albumin, cholesterol, triglycerides, total and direct bilirubin, alkaline phosphatase, serum glutamic–oxalacetic transaminase (SGOT), erythrocyte and leucocyte counts, haematocrit and haemoglobin, as well as osmotic fragility tests after 6 months (Dacie & Lewis, 1968). An Autoanalyzer and standard laboratory procedures were used for the determinations.

At autopsy, the organs were examined grossly, the liver and stomach were weighed and tissues from the myocardium, lungs, liver, stomach, proximal jejunum, distal ileum, descending colon, spleen, kidneys and adrenal glands were fixed in 10% formaldehyde, embedded in paraffin or glycol methacrylate and stained with haematoxylin–eosin.

RESULTS

Table 1 shows that food intake was initially depressed by ATS, but this effect was quickly reversed by the addition of 1% cholesterol to the diet. No deaths occurred spontaneously during the experiment and ATS had no effect on body weight; no differences between the groups were observed at any time. Moreover, the haematological values and osmotic fragility of the erythrocytes were the same in control rats and in animals ingesting ATS. Most of the serum components were unaffected by the ingestion of ATS, but the saponins did reduce plasma cholesterol and triglyceride levels, the differences from the control values being statistically significant ($P < 0.05$ and < 0.02, respectively) at 6 months.

Table 1. *Feed intake in rats fed alfalfa top saponins (ATS)*

Day†	Mean intake (g/day) of diets containing ATS at a level of: 0‡	1%	2%
2	21·4 ± 0·5	20·8 ± 0·7	20·3 ± 0·7
3	29·6 ± 0·9	28·9 ± 0·9	26·5 ± 1·1*
4	31·6 ± 0·8	28·2 ± 1·2*	25·6 ± 1·0**
9	28·9 ± 1·2	31·2 ± 1·7	32·1 ± 0·9
10	30·5 ± 0·9	30·7 ± 1·9	30·6 ± 1·1
11	30·8 ± 1·0	32·0 ± 1·2	30·3 ± 1·0
17	31·0 ± 0·4	31·1 ± 2·9	31·9 ± 1·0
18	30·2 ± 1·0	31·5 ± 1·4	29·9 ± 1·3
19	30·9 ± 1·2	31·4 ± 1·6	31·9 ± 1·2
23	31·7 ± 1·0	31·8 ± 1·3	29·8 ± 1·7
24	29·7 ± 1·0	29·6 ± 1·7	32·1 ± 1·0
53	33·6 ± 1·6	29·0 ± 2·4	33·6 ± 1·9
54	30·9 ± 1·4	30·6 ± 2·0	35·8 ± 0·9

†From day 5, 1% cholesterol was added to each of the diets.

‡Control group.

Values are means ± SEM for groups of eight rats. Those marked with asterisks differ significantly (by Student's *t* test) from the control values: *$P < 0.05$; **$P < 0.001$.

At post-mortem examination, no gross pathological changes were observed. There were no differences in the weights of the liver or stomach of the animals on ATS, and the microscopic findings were similar in all groups, the scattered changes found in the liver, spleen, kidneys and lungs of the ATS-treated rats being found also among the control animals. These changes included an increase in portal mononuclear infiltrate, scattered small mononuclear aggregates in the parenchyma and mild fatty change in the liver, haemosiderin deposits and abundant haemopoiesis in the spleen, and mononuclear infiltrates in the kidney cortex and medulla.

DISCUSSION

In certain respects, ATS are similar to saponins from edible plants that are comparatively harmless when ingested (Shoppe, 1964); humans normally consume without ill effects a number of plants (including alfalfa sprouts, soya beans, chick peas, broad beans, spinach and asparagus), plant extracts (liquorice, sarsparilla and guaiac), infusions (tea), carbonated beverages and other foodstuffs (such as candy and syrups) that contain saponins (Oakenfull, 1981). In contrast, the ingestion of saponins from non-edible plants may be lethal (George, 1965), and saponins are toxic when given intraveneously or when brought into contact with animals with external permeable surfaces, such as snails and fish (Ewart, 1931).

We have demonstrated that in rats fed cholesterol-containing food, the addition of 1 or 2% ATS to the diet is not toxic, as established by food intake, growth and survival rates, multiple serum and haematological determinations (including erythrocyte osmotic fragility), and the gross and histological study of several organs. Differences observed in chicks (Anderson, 1957; Ishaaya *et al.* 1969), in rats studied earlier (Wilcox & Galloway, 1961) and in mice and quail (Reshef *et al.* 1976) may have been species differences or may have been due to the method of preparation of the saponins or to other unidentified factors. However, the results of the study reported here extend those of previous shorter experiments, demonstrating a lack of toxicity of alfalfa saponins given in cholesterol-containing foods to monkeys (Malinow, Connor, McLaughlin, Stafford, Lin, Livingston, Kohler & McNulty, 1981; Malinow, McLaughlin, Kohler & Livingston, 1977). Moreover, ATS seem similar to saponins derived from other sources and shown to be devoid of toxic effects; soya-bean saponins do not depress the growth rate in chicks (Birk, 1969) or in mice (Ishaaya *et al.* 1969), and no adverse effects have been observed in rats fed quillaia saponins (Gaunt, Grasso & Gangolli, 1974; Oser, 1966) or saponin-containing yucca extract (Oser, 1966). Similarly, saponin digitonin has been found to have no ill effects in rats or monkeys (Malinow, McLaughlin & Stafford, 1978).

Alfalfa top saponins lower plasma cholesterol (Malinow *et al.* 1981), decrease intestinal absorption of cholesterol (Malinow *et al.* 1977 & 1981), increase faecal excretion of neutral steroids and bile acids (Malinow *et al.* 1981) and prevent atherosclerosis (Malinow, McLaughlin, Stafford, Livingston & Kohler, 1980). The apparent lack of toxicity of ATS

suggests that these materials may be useful for treating hypercholesterolaemic patients. However, long-term tolerance studies in several species are needed before their use in humans can be advocated.

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HEPATO- AND CARDIOTOXICITY OF *FUSARIUM VERTICILLIOIDES* (*F. MONILIFORME*) ISOLATES FROM SOUTHERN AFRICAN MAIZE

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Abstract—From crops of South African and Transkeian maize were isolated 21 *Fusarium verticillioides* (*F. moniliforme*) strains that do not produce moniliformin, and their toxicity to ducklings and rats was determined. The pathological effects of one strain (MRC 602) were fully characterized and used as a basis for comparison with the other isolates. Acute mortality was common in ducklings fed the isolates, while in rats the mean time to death was at least 24 days even with the most toxic isolate. All the rats fed the MRC 602 isolate died, the mean times to death being 49 and 78 days, respectively, for continual intake of 32 and 16% levels of the fungal-culture material in the diet. Cirrhosis and nodular hyperplasia of the liver, and the occurrence of acute and proliferative endocardial lesions and concurrent intraventricular thrombosis were frequently encountered and were considered to be the most important lesions. Toxic nephrosis, endothelial proliferation in the pulmonary vessels, and thrombosis of the larger vessels in the heart, liver, pancreas, small intestine and lungs were considered to be less important or occurred less often. Lesions considered to be secondary to the above changes were also encountered.

INTRODUCTION

Saccardo (1881) described a fungus found on maize kernels in Italy as *Oospora verticillioides* Sacc. This fungus soon became implicated as the possible cause of pellagra because of its prevalence in maize associated with the disease in Italy (Cuboni, 1882; Tiraboschi, 1905) and in Russia (Deckenbach, 1907). At about the same time, widespread field outbreaks occurred in the United States of a disease in animals associated with the ingestion of mouldy maize; the hooves of cattle and horses sloughed, pigs shed their bristles, chickens lost their feathers, some animals developed convulsions and a high percentage of affected animals died (Peters, 1904). The fungus most commonly associated with the mouldy maize and implicated as the cause of this disease was described by Sheldon (1904) as *Fusarium moniliforme* Sheldon. Although it was soon pointed out that *F. moniliforme* was identical to *O. verticillioides* (Manns & Adams, 1923; Norton & Chen, 1920; Wineland, 1924), the new combination *Fusarium verticillioides* (Sacc.) Nirenberg was made only 5 yr ago (Nirenberg, 1976).

Fusarium verticillioides is now known to be one of the most prevalent seed-borne fungi of maize throughout the world (Booth, 1971; Commonwealth Mycological Institute, 1972). Although this fungus has been suspected of being involved in human and animal diseases since its original description last century, surprisingly little information is available on its toxigenic potential. The available literature on the acute toxicity of *F. verticillioides* to animals and on the toxic metabolites known to be produced by this fungus has been reviewed by Kellerman, Marasas, Pienaar & Naudé (1972), by Kriek, Marasas, Steyn, van Rensburg & Steyn (1977) and by Marasas, Kellerman, Pienaar & Naudé (1976).

The most important mycotoxin known to be elaborated by *F. verticillioides* is moniliformin, which has been reported to be produced by three North American isolates of the fungus (Burmeister, Ciegler & Vesonder, 1979; Cole, Kirksey, Cutler, Doupnik & Peckham, 1973; Springer, Clardy, Cole, Kirksey, Hill, Carlson & Isidor, 1974).

In a study of the toxigenic potential of Fusarium species in South African maize (Marasas, Kriek, Wiggins, Steyn, Towers & Hastie, 1979a), none of 14 toxic isolates of *F. verticillioides* was found to produce moniliformin, compared with 69·6% of 23 toxic isolates of *F. sacchari* (Butl.) Gams var. *subglutinans* (Wr. & Reink.) Nirenberg (alternatively identified as *F. moniliforme* Sheld. var. *subglutinans* Wr. & Reink.). In a study of the incidence of Fusarium species in maize produced in the areas of high oesophageal cancer incidence in Transkei (Marasas, van Rensburg & Mirocha, 1979b), a highly toxic isolate of *F. sacchari* var. *subglutinans* was found to produce up to 11·3 g moniliformin/kg in culture (Kriek *et al.* 1977).

In view of the prevalence of *F. verticillioides* in southern African (i.e. South African and Transkeian) maize (Marasas *et al.* 1979a, b), the absence of moniliformin-producing ability in toxic isolates from maize produced in these areas, and the dearth of information about the pathological lesions induced by *F. verticillioides*, a study was undertaken of the acute and long-term effects of this fungus in experimental animals. This paper reports toxicity studies carried out in ducklings and rats on 21 *F. verticillioides* cultures that were isolated from southern African maize and did not produce moniliformin. The pathology induced by one of the strains (MRC 602) was fully

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characterized and compared with that caused by the other strains.

EXPERIMENTAL

Isolation and culture of fungi. Samples of commercial white dent maize of the 1975 crop were collected in three different geographical areas in the Republic of South Africa, namely at Settlers in the northern Transvaal, at Lichtenburg in the western Transvaal and at Piet Retief in the eastern Transvaal (Marasas *et al.* 1979a). Samples of mouldy home-grown maize ears were also collected during 1975 and 1977 in the Republic of Transkei, in two districts (Lusikisiki and Bizana) in an area where the incidence of oesophageal cancer is low and in two districts (Kentani and Butterworth) in a high-incidence area (Marasas *et al.* 1979b). Fusarium species were isolated from surface-sterilized maize kernels as previously described (Marasas *et al.* 1979a, b). Single-conidial cultures of the 21 isolates of *F. verticillioides* listed in Table 1 were maintained on potato dextrose agar slants. Subcultures from each single-conidial isolate were lyophilized and used to inoculate autoclaved. moistened, whole yellow maize kernels (Kriek *et al.* 1977). The maize cultures were incubated at 25°C for 21 days, dried and ground into a fine meal.

Moniliformin analyses. The isolates of *F. verticillioides* from South African maize (Table 1) were analysed for moniliformin as previously described (Marasas *et al.* 1979a). Moniliformin was determined in the Transkeian isolates (also identified in Table 1) by an HPLC procedure on water extracts of the dried culture material making use of a paired-ion chromatography technique for separation of moniliformin. A reverse-phase column (Partisil 10 ODS, 4 mm × 25 cm) was used with 0·1 M-sodium phosphate buffer (pH 7·0) containing 0·005 M-tetrabutylammonium hydrogen sulphate and 8% methanol as the mobile phase at a flow rate of 1·0 ml/min. The moniliformin was monitored by UV absorption at 229 nm and by comparing the chromatogram with that of authentic pure moniliformin (Thiel, 1979).

Toxicity trials in ducklings. The acute toxicity of culture material of the various isolates of *F. verticillioides* were assayed by feeding diets containing mouldy meal and commercial chicken mash (1 : 1, w/w) *ad lib.* for 14 days to groups of four day-old Pekin ducklings (initial weight *c.* 50 g). Control diets contained 50% maize meal prepared from uninoculated autoclaved yellow maize kernels and 50% commercial chicken mash. The day of death of each duckling was recorded as well as its weight on the day of commencement and on days 7 and 14 of the experiment. Feed intake values were calculated from the initial amount of feed and that remaining at the end of the experiment.

Toxicity trials in rats. Culture material of the different isolates was mixed individually with commerical rat mash to obtain diets containing 8 or 16 and 32%

Table 1. *Area of origin of* F. verticillioides *isolates from southern African maize and toxicity of isolates to Pekin ducklings*

Isolate no. (MRC)	Area of origin	Crop year	Response of treated ducklings: No. of deaths*	Mean time to death (days)†	Total feed intake‡ (g/group)
543	Settlers, SA	1975	2	5·5	600
545	Lichtenburg, SA	1975	4	4·5	20
548	Piet Retief, SA	1975	4	5·7	19
597	Settlers, SA	1975	4	4·7	28
602	Butterworth, Tr.	1975	4	4·5	10
778	Piet Retief, SA	1975	4	4·7	32
785	Lichtenburg, SA	1975	4	6·0	81
815	Lusikisiki, Tr.	1975	4	5·2	31
826	Kentani, Tr.	1975	4	4·2	2
827	Kentani, Tr.	1975	4	5·0	8
828	Kentani, Tr.	1975	4	4·5	5
832	Kentani, Tr.	1975	4	5·5	58
833	Kentani, Tr.	1975	4	4·7	13
834	Kentani, Tr.	1975	4	5·2	22
1044	Lusikisiki, Tr.	1977	4	4·5	14
1052	Butterworth, Tr.	1977	4	5·0	16
1053	Butterworth, Tr.	1977	4	4·7	16
1056	Butterworth, Tr.	1977	4	5·5	14
1060	Bizana, Tr.	1977	4	4·0	22
1064	Bizana, Tr.	1977	4	3·5	3
1069	Kentani, Tr.	1977	2	4·2	424

SA = South Africa Tr. = Transkei

*In groups initially consisting of four ducklings.

†From the start of the experiment.

‡Over the duration of the experiment. Diet containing 50% commercial chicken mash and 50% (w/w) culture material of the appropriate *F. verticillioides* isolate was fed to each group for up to 14 days. Over this period, the control group, in which there were no deaths, consumed 1964 g of feed.

(w/w) mouldy meal. Control diets consisted of 8 or 32% (w/w) ground uninoculated autoclaved yellow maize in rat mash. These diets were fed *ad lib.* to male BD IX rats (initial weight 45 or 75 g). The rats were randomly divided into groups of 20 (for testing MRC 602) and into groups of five or six (for other isolates). The rats were kept two to a cage in a conventional facility (controlled temperature and humidity) with a 12-hr lighting cycle.

Individual weights of the rats were recorded at the start of the experiment and at weekly intervals thereafter. All the rats that died and the survivors, which with the exception of rats treated with MRC 602 were killed after 70 or 77 days of the test feeding by sodium pentobarbital given ip, were autopsied. Specimens of all organs and tissues were collected and preserved in 10% buffered formalin. Conventionally processed haematoxylin and eosin-stained sections were examined by light microscopy.

RESULTS

Mycology

Toxic strains of *F. verticillioides*, which is known to be one of the most prevalent internally seed-borne fungi of maize in southern Africa (Marasas *et al.* 1979a, b), occurred in samples of commercial South African maize as well as in samples of home-grown Transkeian maize.

Moniliformin analyses

The six isolates of *F. verticillioides* from South African maize (Table 1) used in this study have previously been reported to be non-producers of moniliformin (Marasas *et al.* 1979a). Similarly none of the 15 Transkeian isolates produced chemically detectable levels of moniliformin in culture.

Toxicity trials in ducklings

The toxic effects exerted on ducklings by culture material of the 21 non-moniliformin-producing isolates of *F. verticillioides* are summarized in Table 1. All the isolates were acutely toxic to the ducklings and 19 out of the 21 isolates caused the death of all four ducklings in the groups in a mean time of 6 days or less and caused marked reductions in feed intake. Two isolates (MRC 543 and MRC 1069) appeared to be somewhat less toxic, causing the death of only two out of four ducklings; the reduction in feed intake was also less drastic.

Toxicity trials in rats

The toxic effects found in rats fed culture material of *F. verticillioides* strain MRC 602 are summarized in Table 2. Although no acute deaths were recorded, all the rats in both experimental groups died after mean periods of 49 and 78 days for the diets containing 32 and 16% culture material, respectively. Furthermore, the culture material resulted in a markedly reduced weight gain in both experimental groups in comparison with the control group.

The toxicity of the other isolates to rats is recorded in Table 3, which shows a marked variation between the toxicity of the different isolates. Some were noticeably more toxic than MRC 602 while others were not toxic at all at the 32% dietary level.

Gross pathology of MRC 602-treated rats. The changes caused by strain MRC 602 in the rats were studied in detail, and their incidence is tabulated in Table 2. Lesions were observed more consistently in the liver and heart and less often in the other organs

Table 2. *Toxicity of* F. verticillioides *(MRC 602) culture material to male rats*

Parameter or lesion	Findings in rats fed MRC 602* at a dietary level of		
	0 (control)	16%	32%
Initial no. of rats/group	20	20	20
No. of deaths	1	20	20
Mean time to death (days)†	49	78 (44–161)	49 (33–62)
Mean body weight (g) on			
day 0	49	45	45
day 56	253	123	91
day 154	329	102	—
Lesion			
Cirrhosis		20	20
Nodular hyperplasia (liver)		20	15
Cardiac thromboses (ventricular)		15	9
Hydrothorax		13	9
Pulmonary oedema		12	7
Pulmonary emphysema		10	5
Anasarca		11	6
Splenomegaly		14	3
Haematuria		10	7
Fibrino-haemorrhagic enteritis		10	6
Pancreatic necrosis		2	1
Alopecia (bilateral symmetrical)		2	1

*Culture material of *F. verticillioides* (MRC 602) was incorporated at a level of 16 or 32% (w/w) into commercial rat mash and fed *ad lib.* for up to 161 days. The control diet consisted of 32% (w/w) autoclaved maize meal incorporated into commercial rat mash.

†From the start of test feeding; the range of times is given in brackets.

Table 3. *Toxic effects of feeding various isolates of* F. verticillioides *from southern African maize to male rats*

Isolate no.* (MRC)	Dietary level (%) of mouldy‖ meal...	No. of deaths†		Mean time to death (days)‡		Mean weight at death (g)		No. of rats with lesions§ in: Liver		Heart	
		8	32	8	32	8	32	8	32	8	32
—‖		0	0	70	70	303	300	0	0	0	0
543		0	0	77	77	225	216	0	0	0	0
545		0	0	77	77	194	133	0	0	0	0
548		0	5	77	43	191	122	4	5	1	2
597		1	5	58	42	200	85	5	5	0	5
778		0	5	77	49	196	96	4	5	0	1
785		0	5	77	52	223	73	3	5	0	3
815		4	5	67	38	174	80	5	5	1	0
826		5	6	62	44	119	58	6	6	2	6
827		6	6	52	25	116	50	6	¶	1	0
828		1	6	69	40	165	65	6	6	1	1
832		0	6	70	41	203	78	6	6	0	2
833		2	6	67	24	128	64	6	¶	4	1
834		0	0	70	70	254	201	0	0	0	0
1044**		0	2	34	26	135	72	5	5	0	0
1052		5	6	63	28	120	67	6	6	4	0
1053		0	3	70	58	255	109	0	6	0	3
1056		0	6	70	46	213	86	6	6	0	3
1060		4	5	60	31	175	68	5	5	1	0
1064		0	0	77	77	217	151	0	0	0	0
1069		0	0	70	70	239	228	0	0	0	0

*Culture material of each *F. verticillioides* isolate was incorporated at a level of 8 or 32% (w/w) into commercial rat mash and fed *ad lib.* until death or until the experiment was terminated after 70 or 77 days, as indicated.

†In groups consisting initially of six male rats for tests on the Transkeian isolates (nos 815–1069) and five per group for South African isolates (nos 543–785).

‡From the start of test feeding.

§Lesions corresponding to those induced by MRC 602. Those in the liver were toxic hepatosis, cirrhosis and nodular hyperplasia, and the incidence of heart lesions was based exclusively on the presence or absence of intraventricular thrombi.

‖Control diets consisting of 8 or 32% (w/w) autoclaved uninoculated maize meal in rat mash were fed to groups of rats.

¶No distinct microscopic lesions were detectable either because the rats died too soon or because the toxicity of the material was too low.

**This group (fed isolate no. 1044) was killed on day 34, because the food was exhausted.

and tissues. At both dietary levels the lesions were of similar nature but varied in the degree and extent of development. This variation was dependent on the time of death which was related to the dietary levels of the mouldy material. No difference existed between the lesions in the long-surviving rats on the 32% dietary level and those on the 16% dietary level. The rats that died were invariably in a poor condition and showed varying degrees of anaemia and icterus; this became more pronounced in the animals that died later in the experiment.

In the rats dying early in the 32% dietary group (i.e. before day 47) the livers were not visibly cirrhotic. From day 47 onwards, all the livers were cirrhotic, and showed varying degrees of atrophy and the development of varying numbers of hyperplastic nodules. Most of the nodules were small (1–2 mm in diameter) and subcapsular or within the parenchyma. Some of the nodules, however, were very large (up to 1 cm in diameter) and often protruded above the capsular surface, in which case they were either sessile or pedunculated and multilobular.

In the heart, thrombi occurred particularly in the left ventricle. The thrombi varied in size from pinhead to a volume occupying the entire ventricular space. In each case the thrombus was attached only at or near the ventricular apex. Right ventricular and atrial thrombi were rare and were always encountered in conjunction with left ventricular thrombosis.

Histopathology of MRC 602-treated rats. The gross pathological features were confirmed histopathologically. The changes in the two test groups were found to be of a similar nature and varied only in the degree and extent of development. The changes in the liver and heart were considered to be the most consistent.

Liver: Several changes were considered to be characteristic. Extensive fibrosis, resulting initially in a predominantly micronodular cirrhosis, changed into a mixed micro- and macronodular cirrhosis following the development of large hyperplastic nodules (Fig. 1). Marked bile-duct hyperplasia, lipofuscin pigmentation, particularly in the connective-tissue septa, and a scant lymphocytic cell infiltrate (Figs 2 & 3) were other consistent findings. Two distinct populations of hepatocytes were encountered (those outside the hyperplastic nodules and those within them). The former were characterized by a basophilic cytoplasm and occurred as pseudolobules, in clusters or singly within the connective-tissue strands. These cells were rarely organized into trabeculae. Megalocytes were present among them, mitotic figures were rare and the cells were occasionally arranged in alveoli. Within the

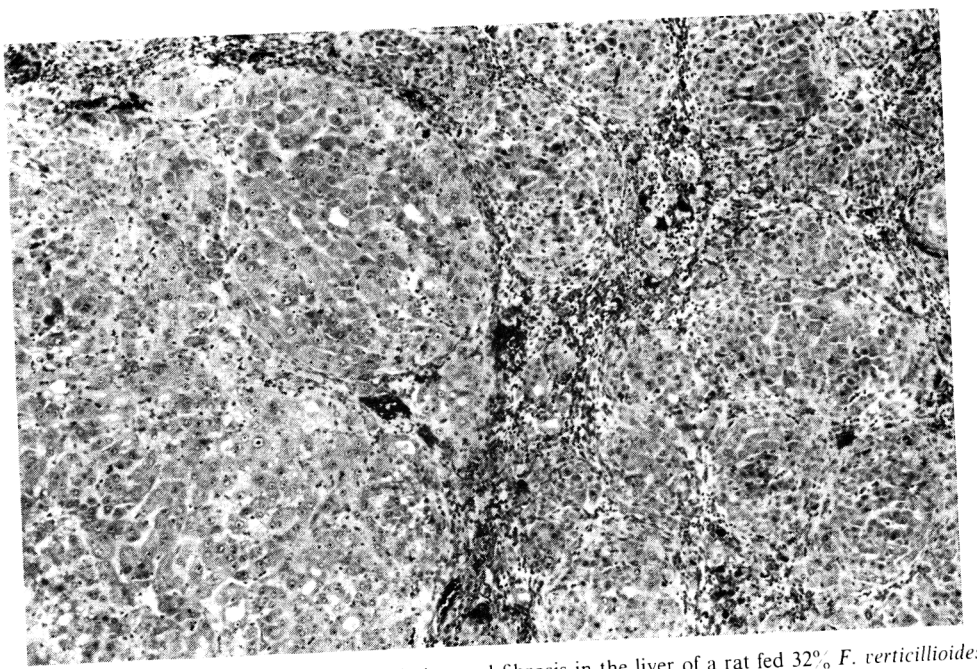

Fig. 1. Nodular hyperplasia, pseudolobulation and fibrosis in the liver of a rat fed 32% *F. verticillioides* in the diet for 62 days. Haematoxylin and eosin (H & E) × 90.

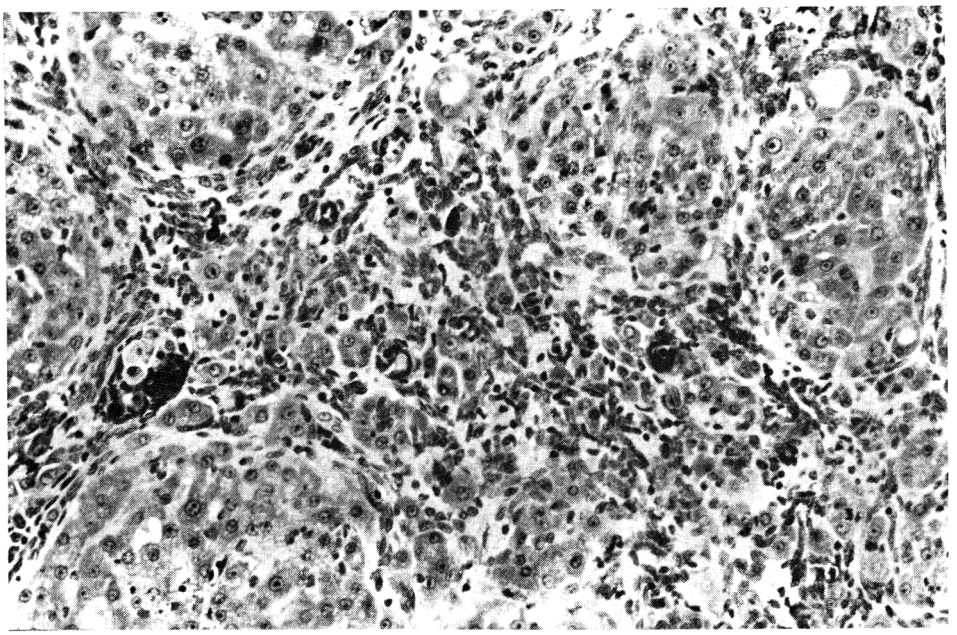

Fig. 2. Pseudolobulation, fibrous tracts enveloping single or small clusters of hepatocytes, and accompanying bile-duct proliferation in a rat fed 32% *F. verticillioides* in the diet for 62 days. H & E × 230.

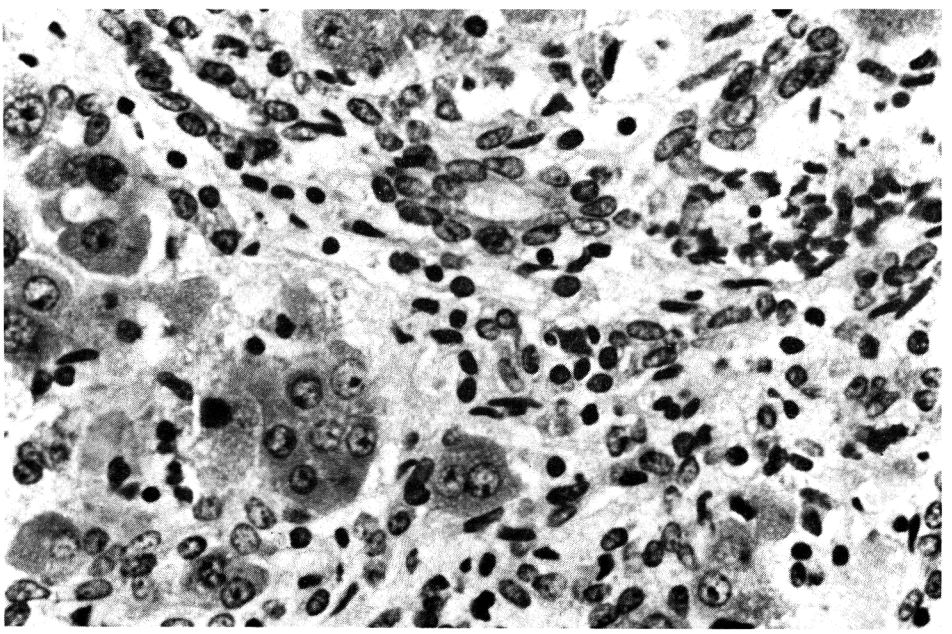

Fig. 3. Bile-duct proliferation, immature fibrous connective tissue and a scant round-cell infiltrate in liver tissue from a rat fed 32% *F. verticillioides* in the diet for 62 days. H & E × 370.

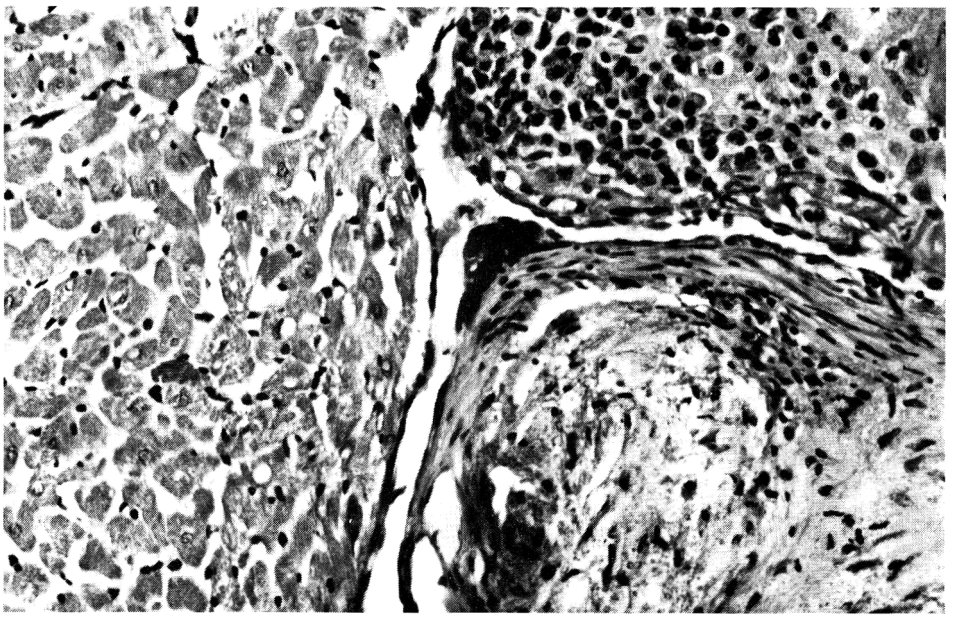

Fig. 4. Organizing thrombus in the left ventricle of the heart of a rat fed 16% *F. verticillioides* in the diet for 77 days. H & E × 290.

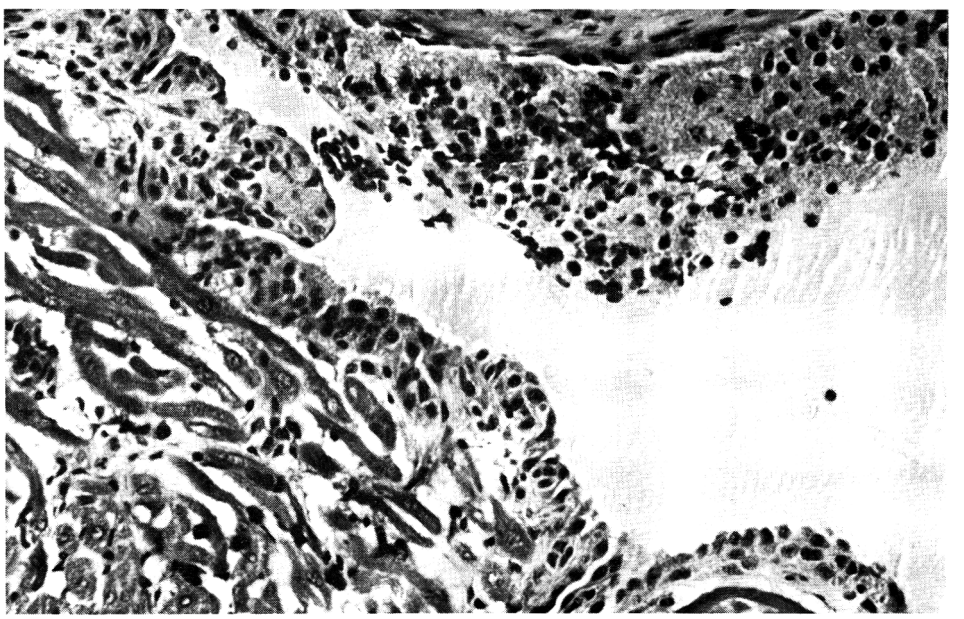

Fig. 5. Endocardial thickening in the left ventricle of the heart as a consequence of endothelial hyperplasia and mild endocardial fibrosis in a rat fed 16% *F. verticillioides* in the diet for 77 days. H & E × 290.

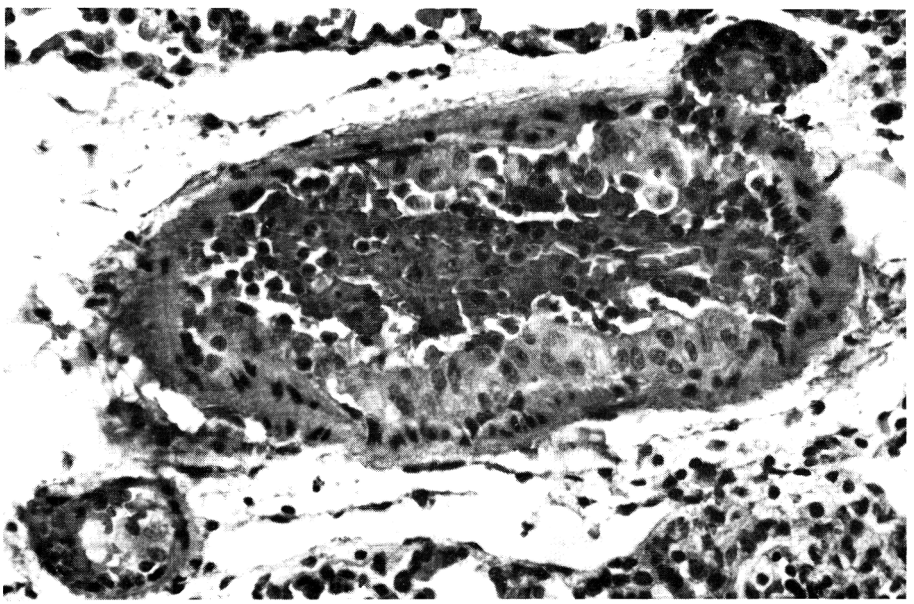

Fig. 6. Prominent endothelial hyperplasia resulting in a circumferential intimal thickening in an intrapulmonary artery of a rat fed 32% *F. ventricillioides* in the diet for 49 days. H & E × 320.

hyperplastic nodules, which were surrounded by connective-tissue strands and were occasionally multilobular, the cells were arranged in well-organized one-cell-thick trabeculae and had a distinct light eosinophilic cytoplasm. Megalocytosis within these nodules was less obvious. A few foci of abnormally proliferating bile ducts, surrounded by a thin connective-tissue capsule (cholangiofibrosis), were encountered. The lumens of these ducts were dilated and lined by cells that were irregular in size, shape and arrangement. The main feature was a clustering of pleomorphic nuclei. Arterial thromboses were rare but were encountered more commonly in the rats that survived longer.

Heart: The cardiac changes encountered were similar in nature and location in the two test groups and varied only in the extent and degree of development. The most prominent changes occurred in the left ventricular endocardium, with lesions occurring more commonly in the middle and apical portions. Depending on the time of death, lesions could be divided into two arbitrary groups, namely early acute lesions in the rats that died before day 50, and proliferative changes in those that died thereafter. Concurrent with the endocardial changes, partially organized ventricular thrombi, varying in size but mostly large, occurred particularly in the left ventricle (Fig. 4).

The early acute lesions in the endocardium were characterized by endothelial swelling, haemorrhage, oedema, macrophage and lymphocytic infiltrates and early fibroplasia. Focal myocardial necrosis with a scant neutrophil infiltrate occurred adjacent to the affected endocardium.

Focal myocytolysis was a rare finding, and extensive myocardial infarction resulting from arterial thrombosis was observed in only one rat. A scant perivascular and interstitial lymphocytic cell infiltrate was common.

The cardiac changes described above were observed less commonly in rats that died after day 50. In these animals the endocardial changes were predominantly proliferative and were characterized by the development of a bi- or multilayered endothelial lining, more prominent endocardial fibroplasia, and the presence of macrophages (Fig. 5). Focal acute myocardial necrosis adjacent to this endocardial involvement was more common than in the early stages, as was myocytolysis. A diffuse but scant round-cell infiltrate often occurred. The ventricular thrombi were in various stages of organization and a few foci of calcification were noted.

Other organs: The changes in the other organs were less commonly encountered and were considered to be less important than those in the liver and heart. Arterial and venous thrombosis, although encountered relatively infrequently, also occurred in the lungs, pancreas and small intestines. Endothelial proliferation was particularly prominent in the larger pulmonary arteries (Fig. 6) and resulted in partial occlusion and occasionally in irregular polypomatous projections into the vascular lumen. Lymphoid atrophy, with occasional necrosis, was a constant finding. Acute pancreatic necrosis was encountered in about 25% of the rats receiving 16% culture material in the diet. An acute necrotic enteritis with a fibrinohaemorrhagic exudate occurred in the animals affected macroscopically. In both the pancreas and small intestine, the lesions commonly occurred in association with extensive thrombosis of either the arteries or veins. A low-grade toxic nephrosis, characterized by varying degrees of necrosis throughout the nephron and a prominent hyaline droplet degeneration, was a constant finding. Testicular degeneration and atrophy, with a total absence of spermatogenesis, occurred in all the rats. Focal adrenocortical necrosis and haemorrhage and focal haemorrhages in the brainstem and cerebellar molecular layer were encountered spasmodically.

Pathology caused by strains other than MRC 602. The gross and microscopic changes caused by the other strains tested (Table 3) corresponded closely to those caused by strain MRC 602. However, two isolates (MRC 545 and 1064) that were acutely toxic to ducklings (Table 1), did not cause any noticeable gross or histological changes in either the heart or the liver of rats. A similar negative effect in rats was encountered with the two isolates (MRC 543 and 1069) that were the least toxic of the isolates tested in ducklings.

DISCUSSION

These studies have demonstrated in ducklings and rats the toxic effects of strains of *F. verticillioides* obtained from commercial and home-grown maize from various regions of South Africa and Transkei. The different isolates showed a marked variation in degree of toxicity, but no basic differences were evident in the types of lesions induced, and it appears that toxigenic strains of this fungus are of common and widespread occurrence.

In rats, hepatotoxicity, endothelial toxicity and, to a lesser extent, nephrotoxicity are considered to be the main effects of the toxin(s) produced by this fungus. Most of the lesions encountered can be explained on the basis of these changes. The importance of the liver changes as the main contributory cause of death is indisputable. Endothelial toxicity manifested by acute exudative or chronic proliferative lesions is the basis of the consistent intraventricular thrombosis found in the heart as well as of the less consistent vascular changes and thrombosis encountered elsewhere. These lesions were no doubt also contributory causes of death.

The insidious nature of the development of the lesions in rats was unexpected. Even with the most toxic strain the mean times of death were 52 and 25 days for the rats fed 8 and 32% dietary concentrations of the culture material, respectively (Table 3). This is of relevance in assessing the toxicity of similar strains of this fungus; acute/short-term toxicity trials will be inconclusive and will most probably be negative. Despite the relatively slow development of the lesions, 100% mortality can be expected when toxic strains are fed for a prolonged period. Moreover, while the isolates were more acutely toxic to ducklings, this toxicity to ducklings was not consistently associated with hepatic and cardiac lesions in rats. Ducklings are thus an unsuitable bioassay system for the screening of hepato- and cardiotoxic strains of *F. verticillioides*.

While specific involvement of the myocardium (focal Zenker's necrosis, myolysis and fibrosis) is a feature of the chronic lesions caused in rats by moniliformin (Kriek *et al.* 1977), the target cells in the case of cardiotoxicity caused by *F. verticillioides* strains that do not produce moniliformin appear to be endothelial cells, with only rare secondary involvement of the myocardium. Furthermore, hepatotoxicity is not a feature of chronic moniliformin intoxication. Morphological observations, therefore, indicate no relationship between the manifestations of toxicity of the strains of *F. verticillioides* that produce moniliformin and those that do not.

Extrapolation between species is dangerous, but the possible involvement of metabolites produced by this widespread fungus in the aetiology of unexplained toxic hepatoses in both man and animals and in the aetiology of certain types of ideopathic cardiomyopathy in man should be examined.

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DISPOSITION OF [^{14}C]CAPROLACTAM IN THE RAT

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Abstract—The tissue distribution and excretion of [*carbonyl*-^{14}C]caprolactam was studied in male Fischer 344 rats given a single oral dose of 0·18 mg/kg body weight. After 24 hr, 77·6% of the administered radioactivity had been excreted in the urine, 3·5% in the faeces and 1·5% in the expired air of the animals. Elimination of radioactivity in the urine and expired air was most rapid during the initial 6 hr following dosing. Analysis of the urine by high-pressure liquid chromatography indicated that after 24 hr, only 2·3% of the excreted radioactivity was in the form of the parent compound. Two major urinary metabolites of caprolactam (MI and MII) were detected comprising 79·3% and 17·7% of the excreted radioactivity, respectively, in the 24-hr urine. The disposition of an oral dose of 1·5 g [^{14}C]-caprolactam/kg was also determined. The pattern of tissue distribution of radioactivity 6 hr after administration of the high dose of caprolactam differed little from that observed after the low dose, except that 42% of the administered radioactivity remained in the stomach of animals given 1·5 g/kg body weight, compared with only 6% in the stomach of animals given 0·18 mg/kg body weight. After 6 hr, 39% of the administered radioactivity had been excreted in the urine of the low-dose group whereas only 14% of the administered radioactivity was found in the urine of the high-dose group. Pretreatment with 1·5 g caprolactam/kg body weight/day for 7 days had little effect on the tissue distribution and urinary and faecal excretion of an oral dose of 1·5 g [^{14}C]caprolactam/kg. However, a five-fold increase in the excretion of radioactivity in the expired air of the pretreated animals (0·25% of administered radioactivity at 6 hr) was observed.

INTRODUCTION

Caprolactam is the monomer used in the manufacture of the polymer Nylon 6. In the synthesis of Nylon 6, caprolactam is first hydrolysed to the straight-chain ε-aminocaproic acid which then polymerizes *via* peptide linkages to the linear polymer. Nylon 6 is used in the production of tyre cord, clothing, carpeting, plastics and food packaging, and has recently been approved by the US Food and Drug Administration for use in food-contact films. US production of the polymer is estimated at over $0{\cdot}5 \times 10^9$ kg/year.

Acute oral LD_{50} values in excess of 1 g/kg body weight in the rat (Bornmann & Loeser, 1959; Lomonova & Preobrazenskaya, 1961; Smyth, Carpenter, Weil, Pozzani, Striegel & Nycum, 1969) and rabbit (Lomonova & Preobrazenskaya, 1961) have been reported. Hohensee (1951) reported the LD_{50} values of caprolactam in the mouse as 0·75 g/kg (sc), 0·58 g/kg (ip) and 0·48 g/kg (iv); the smallest oral dose resulting in 100% mortality in the mouse was 1·2 g/kg. Other acute effects attributed to caprolactam include skin irritation in man (Dovzhanskii, Suvorov, Ardentov, Granovskii, Dolzhikov & Khonin, 1972) and sensitization in guinea-pigs (Ivanova, Statgek, Nyachenko & Doda, 1973). Toxic effects observed after an acute lethal dose of caprolactam in the rat included stupor and bleeding from the nostrils, followed by clonic convulsions which progressed to tonic convulsions and death (Goldblatt, Farquharson, Bennett & Askew, 1954). Chronic and subchronic administration of caprolactam to rodents did not produce pathological changes (Ceresa & Graziloi, 1952; Filippov, Kovalenko & Ermakova, 1975; Goldblatt *et al.* 1954). Greene, Friedman & Sherrod (1979), using a variety of bacterial and mammalian cell screens, have recently reported that caprolactam shows no mutagenic activity. A bioassay recently conducted for the US National Cancer Institute indicated that caprolactam was not carcinogenic for Fischer 344 rats or B6C3F1 mice (US Department of Health and Human Services, 1980).

In a 28-day study, caprolactam added to the diet of weanling male and female rats at levels of 0, 0·2, 1·0 and 5% resulted in a dose-related decrease in food consumption and weight gain (Wijnands & Feron, 1969). However, adaptation to caprolactam in the diet has been observed. Upon introduction of caprolactam to the diet, weanling rats exhibited initial growth suppression followed 3–5 days later by an increase in food intake and rate of weight gain rivalling that of control animals (Morrison, Ross & Ruth, 1980).

In view of the large quantities of caprolactam produced and its potential capacity to contaminate the environment, and since no data on metabolism were available, the present study was undertaken to determine the tissue distribution and excretion of caprolactam.

EXPERIMENTAL

Tissue distribution after a single oral dose of 0·18 mg [^{14}C]caprolactam/kg. Male Fischer 344 rats (Charles River Breeding Laboratory, Wilmington, MA), weighing 124 ± 19 g (mean ± SD) were used. [*Carbonyl*-^{14}C]caprolactam with a specific activity of 5·32 mCi/mmol was obtained from New England Nuclear (Boston, MA). Radiochemical purity as determined by thin-layer chromatography and high-pressure liquid chromatography (Unger & Friedman,

1980) was greater than 99%. Labelled caprolactam was diluted with unlabelled compound to yield a mixture containing 0·143 mg/ml and 6·8 μCi/ml. The solvent vehicle was distilled water. The concentration of caprolactam in the dosing solution was verified by high-pressure liquid chromatography and the specific activity by liquid scintillation spectroscopy. The rats were dosed by oral intubation with 1 μCi [^{14}C]caprolactam (0·18 ± 0·018 mg/kg, mean ±SD), and killed with ether after 0·5, 1, 2, 3, 4, 6, 15 or 24 hr (five animals at a time). The animals were housed individually in glass Roth-type metabolism cages (Bellacour Company, Laurelton, NY) which allowed separate collection of urine and faeces; expired carbon dioxide was trapped in ethanolamine. Organ and tissue samples were taken at autopsy for quantitation of radioactivity. Urine was collected on ice at the intervals indicated above. The contents of the urinary bladder were combined with the urine collected from each animal. The residual radioactivity in the urinary collection funnels of the metabolism cages was rinsed with water into separate containers and the radioactivity determined. The radioactivity in the faecal samples was also determined.

Effect of caprolactam pretreatment on the tissue distribution of 1·5 g [^{14}C]caprolactam/kg. For this study, labelled caprolactam was diluted with unlabelled caprolactam to yield a mixture containing 0·48 g/ml and 1·13 μCi/ml. Five male Fischer 344 rats were dosed by oral intubation with 0·57 μCi [^{14}C]caprolactam (1·5 g/kg), killed with ether after 6 hr and their tissues, blood, urine, faeces and expired carbon dioxide were collected for determination of radioactivity. In addition, a separate group of six rats was treated with 1·5 g/kg unlabelled caprolactam orally each day for 7 days. At the end of the 7-day period (24 hr after the last dose of unlabelled caprolactam), the animals were dosed orally with 1·5 g [^{14}C]caprolactam/kg, killed after 6 hr and their tissues, blood, urine, faeces and expired carbon dioxide were collected for determination of radioactivity. A further three animals were dosed with 1·5 g [^{14}C]caprolactam/kg and their urines were collected after 24 hr for determination of caprolactam and its metabolites.

Determination of caprolactam and its metabolites in urine. The 6- and 24-hr urine samples collected from animals given 0·18 mg and 1·5 g [^{14}C]caprolactam/kg were subjected to analysis by high-pressure liquid chromatography (HPLC; Unger & Friedman, 1980). The urine samples were centrifuged to remove particulate matter and injected directly onto a Laboratory Data Control (Riviera Beach, FL, USA) HPLC system. Components were separated on two microparticulate reversed-phase columns (Lion Technology, Dover, NJ) with acetonitrile–water (11:89, v/v) as the elution solvent. Caprolactam was detected by UV absorbance at 210 nm. Metabolites were quantitated by determination of radioactivity in fractions collected from the HPLC eluate.

Determination of radioactivity. Blood and tissue samples were weighed in cellulose cones and combusted in a Packard Tri-Carb model B306 sample oxidizer (Packard Instrument Co., Inc., Downers Grove, IL). All the faeces from each animal were divided among cellulose cones and combusted. The stomach and small and large intestine were combusted along with their contents. Aliquots of the urine samples were mixed with 10 ml Aquasol-2 (New England Nuclear) before counting. Aliquots of the carbon dioxide-trapping solution (1 ml) were mixed with 4 ml methanol and 5 ml Permafluor (Packard Instrument Co.) before counting. Radioactivity was determined in a Beckman LS 9000 liquid scintillation spectrometer (Beckman Instruments, Inc., Fullerton, CA). Sample quench was compensated for by the use of quench curves and external standardization.

RESULTS

Radioactivity in the urine, faeces and expired air of animals given 0·18 mg [^{14}C]caprolactam/kg

Twenty-four hours after administration of an oral dose of 0·18 mg/kg [^{14}C]caprolactam, 77·6 ± 0·7% (mean ± SEM) of the administered radioactivity had been excreted in the urine, 3·5 ± 1·1% in the faeces and 1·5 ± 0·1% in the expired air of the animals. The cumulative excretion of radioactivity by these routes is shown in Fig. 1. Elimination of radioactivity in the urine and expired air was most rapid during the initial 6 hr following dosing, after which excretion continued at a much reduced rate.

Tissue distribution after a single oral dose of 0·18 mg [^{14}C]caprolactam/kg

The concentrations of radioactivity in the tissues of the rats following the administration of 0·18 mg [^{14}C]caprolactam/kg are shown in Table 1. With the exception of the stomach, bladder, kidneys and fat, the concentrations of radioactivity in the tissues were similar to that in the blood. The concentration of

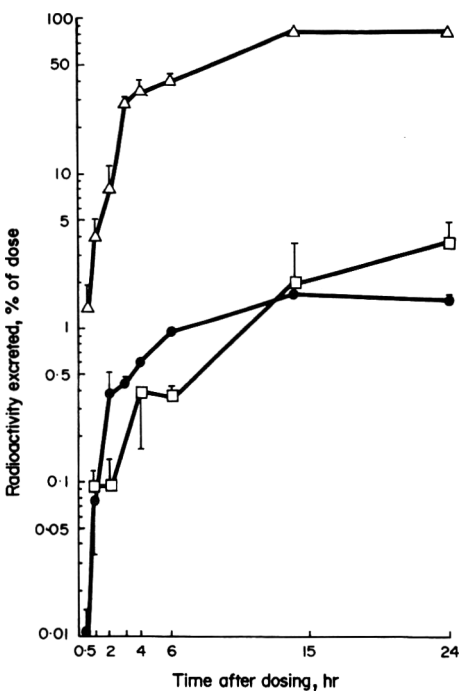

Fig. 1. Cumulative excretion of [^{14}C]caprolactam-derived radioactivity in the urine (△), faeces (□) and expired air (●) of rats after a single oral dose of 0·18 mg [^{14}C]caprolactam/kg. Results are the means (±SEM) of five determinations.

Table 1. *The distribution of radioactivity in the tissues of male rats at intervals following the oral administration of 0·18 mg [^{14}C]caprolactam/kg body weight*

Tissue	Time after dosing (hr)...	Distribution of radioactivity (ng equivalents caprolactam/g tissue)							
		0·5	1	2	3	4	6	15	24
Liver		104 ± 12	151 ± 13	96 ± 9	70 ± 6	60 ± 5	49 ± 9	3·2 ± 1	2 ± 0·1
Spleen		91 ± 8	140 ± 12	100 ± 7	71 ± 7	60 ± 4	44 ± 7	1·6 ± 0·1	1 ± 0·1
Stomach*		1907 ± 286	1538 ± 397	735 ± 196	931 ± 235	719 ± 258	690 ± 135	1·7 ± 0·1	0·7 ± 0·1
Small intestine*		119 ± 9	158 ± 9	110 ± 5	85 ± 7	67 ± 6	54 ± 9	2·9 ± 0·2	1·2 ± 0·1
Large intestine*		64 ± 6	93 ± 3	77 ± 2	72 ± 6	66 ± 7	64 ± 5	37 ± 9	8 ± 0·9
Pancreas		101 ± 9	116 ± 4	78 ± 9	58 ± 5	50 ± 6	35 ± 6	1·5 ± 0·1	2 ± 1·3
Kidneys		130 ± 13	247 ± 19	189 ± 5	171 ± 9	146 ± 18	105 ± 18	4·1 ± 0·2	1·4 ± 0·02
Bladder		378 ± 155	1240 ± 223	1136 ± 287	1201 ± 230	573 ± 235	797 ± 192	56 ± 13	—
Adrenals		74 ± 16	162 ± 35	70 ± 6	59 ± 7	61 ± 9	41 ± 7	—†	—
Testes		75 ± 8	93 ± 13	77 ± 2	63 ± 10	59 ± 4	44 ± 6	6·7 ± 0·2	1·1 ± 0·3
Epididymis		72 ± 9	112 ± 16	77 ± 3	57 ± 4	51 ± 2	41 ± 6	3 ± 0·2	—
Thyroid		75 ± 18	155 ± 48	93 ± 17	84 ± 15	79 ± 2	38 ± 4	—	—
Pituitary		115 ± 35	146 ± 28	104 ± 6	73 ± 14	48 ± 19	—	—	—
Thymus		85 ± 10	128 ± 13	86 ± 5	58 ± 5	49 ± 3	37 ± 6	2 ± 0·1	—
Salivary gland		77 ± 9	114 ± 10	85 ± 4	53 ± 4	45 ± 4	36 ± 6	2 ± 0·1	0·6 ± 0·03
Lymph nodes‡		66 ± 17	90 ± 6	62 ± 6	54 ± 3	57 ± 6	32 ± 6	—	—
Heart		91 ± 10	116 ± 4	90 ± 5	58 ± 6	48 ± 4	38 ± 7	1·3 ± 0·1	0·4 ± 0·1
Aorta		100 ± 12	131 ± 10	79 ± 4	79 ± 7	79 ± 18	39 ± 7	—	—
Lungs		78 ± 16	135 ± 11	93 ± 5	63 ± 6	52 ± 4	46 ± 8	2·1 ± 0·1	0·9 ± 0·1
Sternum		68 ± 6	106 ± 10	73 ± 3	52 ± 4	42 ± 3	30 ± 6	1·3 ± 0·1	0·6 ± 0·1
Muscle		75 ± 8	112 ± 11	78 ± 3	54 ± 4	46 ± 3	35 ± 5	1·1 ± 0·1	0·3 ± 0·04
Brain		69 ± 8	81 ± 8	77 ± 4	54 ± 5	47 ± 3	37 ± 6	1·4 ± 0·1	0·4 ± 0·05
Eyes		69 ± 7	103 ± 3	82 ± 3	69 ± 6	58 ± 4	48 ± 6	3·3 ± 0·3	1·9 ± 0·1
Skin		71 ± 9	107 ± 8	74 ± 3	57 ± 5	47 ± 4	42 ± 7	2·6 ± 0·1	1·1 ± 0·1
Fat§		18 ± 2	41 ± 7	15 ± 4	21 ± 2	25 ± 3	13 ± 1	1·5 ± 0·1	2·4 ± 0·2
Blood		88 ± 9	128 ± 9	95 ± 6	62 ± 6	53 ± 4	40 ± 7	2·6 ± 0·9	0·7 ± 0·1

Values are the means ± SEM of five determinations.
*Including contents.
†The level of radioactivity was too low for accurate measurement.
‡Lymph nodes were excised from the serosal surface of the small intestine.
§Fat was taken from the supratesticular fat pad.

Table 2. *The distribution of radioactivity in the tissues of non-pretreated (control) and pretreated male rats 6 hr following the oral administration of 1·5 g caprolactam/kg body weight*

Tissue	Distribution of radioactivity (mg equivalents caprolactam/g tissue)	
	Control rats	Pretreated rats
Liver	0·34 ± 0·10	0·60 ± 0·09
Spleen	0·39 ± 0·07	0·57 ± 0·09
Stomach†	17·85 ± 4·27	22·81 ± 3·66
Small intestine†	0·51 ± 0·08	0·76 ± 0·09
Large intestine†	0·44 ± 0·04	0·69 ± 0·11
Pancreas	0·35 ± 0·04	0·48 ± 0·07
Kidneys	0·54 ± 0·07	0·91* ± 0·12
Bladder	2·01 ± 0·38	3·99 ± 1·19
Adrenals	0·32 ± 0·05	0·48 ± 0·09
Testes	0·44 ± 0·05	0·62 ± 0·17
Epididymis	0·37 ± 0·04	0·51 ± 0·07
Thyroid	0·33 ± 0·08	0·50 ± 0·09
Thymus	0·38 ± 0·05	0·53 ± 0·08
Salivary gland	0·38 ± 0·04	0·49 ± 0·08
Lymph nodes‡	0·26 ± 0·03	0·32 ± 0·04
Heart	0·41 ± 0·05	0·56 ± 0·09
Aorta	0·34 ± 0·04	0·53* ± 0·05
Lungs	0·40 ± 0·03	0·56 ± 0·10
Sternum	0·34 ± 0·04	0·47 ± 0·08
Muscle	0·39 ± 0·05	0·52 ± 0·08
Brain	0·41 ± 0·05	0·48 ± 0·07
Eyes	0·41 ± 0·06	0·58 ± 0·10
Skin	0·32 ± 0·04	0·41 ± 0·06
Fat§	0·07 ± 0·01	0·13* ± 0·02
Blood	0·42 ± 0·05	0·58 ± 0·09

Values are the means (±SEM) of five or six determinations. Those marked with asterisks differed significantly ($P < 0·05$) from the control values (Student's t test). The percentage increases shown by the significant values were 69% (kidneys), 57% (aorta) and 75% (fat).

†Including contents.

‡The lymph nodes were excised from the serosal surface of the intestine.

§Fat was taken from the supratesticular fat pad.

radioactivity in the fat was consistently lower than that in the blood or other tissues, indicating a low affinity of adipose tissue for caprolactam and/or its metabolites. Disappearance of radioactivity from the stomach was monophasic and followed first-order kinetics ($r > 0·96$) with an elimination half-life of 1·87 hr. Correlation coefficients were determined from least-squares linear regression analysis of the data. The high concentration of radioactivity found in the stomach was not surprising, since the animals were orally dosed. However, the concentration of radioactivity in the small intestine generally exceeded that of the blood by less than 40%. The increase was statistically significant only at the 0·5, 1·0, 3·0 and 24 hr observations (Student's t-test, $P < 0·05$). Also, except at the latter times, the concentration of radioactivity in the small intestine never approached that in the stomach.

The high concentrations of radioactivity observed in the kidneys and bladder reflect the importance of the urine as a route of excretion. The concentration of radioactivity in the liver did not significantly exceed that in the blood (Student's t-test, $P < 0·05$), except after 24 hr. With the exception of the stomach, the concentration of radioactivity in all tissues was greatest 1 hr after dosing. Subsequently, the decrease in concentration of radioactivity in all tissues except the large intestine was monophasic and followed first-order kinetics. The half-life of disappearance of radioactivity from the blood was 2·98 hr ($r > 0·99$).

Effect of caprolactam pretreatment on the tissue distribution of 1·5 g [^{14}C]caprolactam/kg

The tissue distribution of radioactivity 6 hr after an oral dose of 1·5 g [^{14}C]caprolactam/kg is shown in Table 2. The pattern of distribution of radioactivity in animals given 1·5 g [^{14}C]caprolactam/kg was similar to that of animals given 0·18 mg/kg, with most of the tissue concentrations approximating that of the blood. The concentration of radioactivity in the fat of animals given 1·5 g/kg was (as in animals given the low dose of caprolactam) several-fold less than in the other tissues. The concentration of radioactivity in the stomach relative to the concentration in the blood and other tissues was much higher after the high dose of [^{14}C]caprolactam than after the low dose. Six hours after administration of the high dose, 42·3 ± 8·2% (mean ± SEM) of the administered radioactivity remained in the stomach, whereas after 6 hr only 6·2 ± 1·5% of the low dose was found in the

Table 3. *Caprolactam and its two major metabolites in the urine of rats dosed orally with 0·18 mg or 1·5 g [^{14}C]caprolactam/kg body weight*

Component	Time after dosing (hr)...	Percentage of total urinary radioactivity			
		0·18 mg caprolactam/kg		1·5 g caprolactam/kg	
		6	24	6	24
Metabolite I (MI)		78·1 ± 3·6	79·3 ± 2·4	25·1 ± 6·0	59·5 ± 4·8
Metabolite II (MII)		16·9 ± 3·7	17·7 ± 2·1	14·1 ± 6·4	15·7 ± 5·7
Parent Compound		3·9 ± 1·2	2·3 ± 0·6	55·2 ± 7·2	14·7 ± 3·5

Values are means ± SD for three determinations.

stomach. This difference was reflected in the amount of radioactivity excreted in the urine. After 6 hr 38·6 ± 3·5% of the administered radioactivity had been excreted in the urine of the animals given 0·18 mg/kg, compared with 13·8 ± 1·3% in the animals given 1·5 g/kg. No significant difference was noted in the percentage of administered radioactivity excreted in the faeces between the two dose groups.

The seven-day pretreatment with caprolactam had little effect on the tissue concentrations of radioactivity (Table 2). A large difference was noted, however, in the percentage of administered radioactivity excreted in the expired air. After pretreatment, 0·25 ± 0·01% (mean ± SEM) of the administered radioactivity was excreted *via* this route, representing a five-fold increase over the percentage excreted by the same route in non-pretreated animals (0·05 ± 0·02%). No significant difference was noted in the urinary and faecal excretion of radioactivity between pretreated and non-pretreated animals (Student's *t*-test, $P < 0·05$).

HPLC analysis of the urine

As shown in Table 3, 78% of the radioactivity excreted in the urine 6 hr after an oral dose of 0·18 mg/kg [^{14}C]caprolactam was in the form of a major metabolite (MI). A lesser quantity of another metabolite (MII) was also found. The relative proportions of MI, MII, and the parent compound were not significantly different in the 24-hr urine. In contrast, 6 hr following an oral dose of 1·5 g/kg, 55·2% of the excreted radioactivity was found as the parent compound, with 25·1% of the radioactivity in the form of the major metabolite. By 24 hr, however, 59·5% of the excreted radioactivity was in the form of the MI metabolite, with 14·7% as the parent compound.

DISCUSSION

The results of these studies indicate that caprolactam, administered orally, is rapidly absorbed, and rapidly eliminated mainly by urinary excretion. The data in Table 1 indicate that [^{14}C]caprolactam-derived radioactivity was cleared from the tissues monoexponentially; there was no evidence of a well-defined tissue-distribution phase. Such a pattern would be expected where the rate of elimination approaches the rate of tissue absorption.

The pattern of distribution of radioactivity in the gastro-intestinal tract after dosing suggests that [^{14}C]caprolactam might be absorbed from the stomach. Absorption of caprolactam from the stomach would be consistent with the pattern of distribution and excretion of [^{14}C]caprolactam-derived radioactivity observed in the present study. The concentration of radioactivity in the small intestine never exceeded that of the blood by more than 35–40% except after 24 hr, and at all except the 15 and 24 hr time points the concentration of radioactivity in the stomach was approximately ten times that in the small intestine (Table 1). Gastric absorption of caprolactam would result in rapid achievement of peak levels in the central compartment and would therefore lead to rapid metabolism and/or excretion. There are a number of possible explanations of the difference between the percentage of administered radioactivity remaining in the stomach 6 hr after the high and the low doses of [^{14}C]caprolactam. It is likely that the rate-limiting step in the absorption of caprolactam (passive absorption of caprolactam from the gastric contents to the systemic circulation) after administration of the high dose was overloaded. Acid-trapping of basic metabolites (of which there is no evidence at present) or precipitation of caprolactam are alternative explanations.

Except after 24 hr, the concentration of radioactivity in the liver of animals given 0·18 mg/kg never significantly ($P < 0·05$) exceeded that in the blood (Table 1). This observation was surprising, since the liver is the organ usually most involved in the biotransformation of xenobiotic compounds and therefore might be expected to accumulate relatively high levels of radioactivity derived from the parent compound. The absence, in the present study, of high concentrations of radioactivity in the liver relative to the blood and other tissues suggests that the liver plays a limited (although not necessarily unimportant) role in the disposition of caprolactam. The results of the present study also indicate that extensive biotransformation is not essential for the elimination of caprolactam, since after administration of the high dose of caprolactam an appreciable quantity of the radioactivity eliminated in the urine took the form of the parent compound (Table 3).

Caprolactam has been shown to produce a transient increase in hepatic tyrosine aminotransferase (TAT) and tryptophan oxygenase (TPO) activities in the rat (Friedman & Salerno, 1980). Maximum induction was noted 6 hr after oral administration of 1·5 g caprolactam/kg body weight; after 24 hr TAT and TPO activities were still significantly elevated over control values. After seven days of daily administration to rats of 0·5 g caprolactam/kg, hepatic TAT and TPO activities had returned to control values. The

role (if any) of hepatic enzyme induction in the five-fold increase in radioactivity excreted in the expired air of the pretreated animals observed in the present study, however, remains unclear.

After administration of 0·18 mg [^{14}C]caprolactam/kg, urinary excretion of radioactivity was mainly in the form of an unidentified metabolite (Table 3). However, after administration of the higher dose of [^{14}C]caprolactam, a far greater percentage of the radioactivity was excreted as the parent compound. This shift in the pattern of urinary excretion indicates that the biotransformation pathway responsible for the metabolism of caprolactam became saturated after exposure to the high level of caprolactam.

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ACUTE STRUCTURAL CHANGES IN RENAL TUBULAR EPITHELIUM FOLLOWING ADMINISTRATION OF NITRILOTRIACETATE

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Abstract—The acute effects of NTA on kidney structure in male, Sprague–Dawley rats were assessed by light microscopy in a series of dose-response and time-course experiments. Focal cytoplasmic vacuolation of the proximal tubule cell was the only treatment-related alteration of kidney structure observed. Three grades of this vacuolar lesion were distinguished over the range of NTA doses (0·11 to 7·3 mmol/kg) that induced this response 6 hr after treatment. No change in tubule structure was observed at the 0·073 mmol/kg NTA dose level. The vacuolar lesion developed between 1·5 and 6·0 hr after treatment with a 7·3 mmol/kg dose of NTA. All the vacuolar lesions were reversible. The most severe vacuolar lesions persisted for up to 72 hr after treatment.

INTRODUCTION

Trisodium nitrilotriacetate monohydrate ($Na_3NTA.H_2O$) is an effective chelating agent that has been used as a builder in detergent formulations (Pollard, 1966). When administered orally, $Na_3NTA.H_2O$ or Na_2HNTA is well absorbed (40 to 90%) by mice, rats and dogs, but less well absorbed (12 to 25%) by rabbits, monkeys and man (Anderson & Kanerva, 1978; Budny, 1972; Budny & Arnold, 1973; Chu, Becking, Villeneuve & Viau, 1978; Michael & Wakim, 1971; Tjalve, 1972). In all the species examined it has been found that, except for the exchange of cations, absorbed nitrilotriacetate (NTA) does not undergo any detectable biotransformation *in vivo* (Budny, 1972; Budny & Arnold, 1973; Michael & Wakim, 1971). These same studies also showed that following absorption NTA is excreted exclusively in the urine.

Various forms of dose-related nephrotoxicity have been reported in test animals that received high doses of $Na_3NTA.H_2O$ either in their food or water for periods ranging from 4 wk to 2 yr. Haematuria, glucosuria and the presence of crystalline CaNaNTA in the voided urine are signs of renal dysfunction that have been observed in rats given a diet containing 2% $Na_3NTA.H_2O$ or water containing 1% $Na_3NTA.H_2O$ for 4 wk (Anderson & Kanerva, 1979; Mahaffey & Goyer, 1972). Alterations in urinary tract tissues, including cytoplasmic vacuolation of proximal tubules, hydronephrosis and erosion and ulceration of the pelvic transitional epithelium have been reported in Sprague–Dawley rats that received NTA at levels of 0·15% or more of their diet (Alden, Kanerva & Anderson, 1981; Mahaffey & Goyer, 1972; Nixon, 1971; Nixon, Buehler & Niewenhuis, 1972). However, when a diet containing 0·03% $Na_3NTA.H_2O$ was administered to Sprague–Dawley rats for 2 yr no treatment-related changes in urinary-tract tissues were found (Nixon *et al.* 1972). Similar results were also noted when Fischer 344 rats were administered NTA in their diet for up to 2 yr. At levels up to 0·2% of the diet NTA did not induce urinary tract lesions. However, both hydronephrosis and nephritis were observed in rats that received NTA at doses of 0·75% or more of their diet (DHEW Publication, 1977).

The development of urinary tract tumours has been observed in rats and mice fed diets containing nephrotoxic levels ($\geq$0·15% of diet) of either $Na_3NTA.H_2O$ or H_3NTA for 18 to 24 months (C. L. Alden, personal communication, 1980; DHEW Publication, 1977). When fed levels of NTA which are not associated with other forms of nephrotoxicity, however, tumour development was not observed in test animals despite exposure for periods of up to 2 yr (Nixon *et al.* 1972; DHEW Publication, 1977). These results suggest that NTA-associated renal tumours may develop as a direct result of a local effect which can only occur when levels of NTA are high enough to induce nephrotoxicity.

The lesions which develop following the administration of NTA have previously been described (Mahaffey & Goyer, 1972; Nixon, 1971; Nixon *et al.* 1972). However, a concerted effort has been made to learn more about the genesis of the non-neoplastic, NTA-associated renal lesions and to determine the role of these lesions in the tumorigenic process. The present study was carried out to provide information on the acute effects of NTA on kidney structure. In particular, the amount of NTA necessary to induce such structural changes and the time necessary for these changes to develop were examined.

EXPERIMENTAL

Mature, male Sprague–Dawley rats (Charles River Breeding Laboratories, Wilmington, MA) were used in all experiments. On the day of the experiment the rats were marked for identification and weighed. Animal weights ranged from 150 to 350 g. The rats were housed individually in stainless steel cages during the

experimental period. Free access to Purina Rat Chow and distilled water was permitted for at least one day before the start of the experiment and during the experimental period.

All the NTA solutions were prepared by dissolving commercial grade $Na_3NTA.H_2O$ (mol wt 275; Monsanto Chemical Co., St. Louis, MO) in deionized-distilled water. The pH of this solution was adjusted to 8·5 by the addition of concentrated HCl. Depending on the dose to be administered, the final concentration of the NTA-solution was either 0·9 or 0·09 mmol/ml. All the solutions were administered by gavage. Except as noted below, the rats in all the time-course experiments received 7·3 mmol NTA/kg body weight. Groups of two to four animals were killed 1·5, 6·0, 18, 24, 48 and 72 hr after dosing. In the dose-response experiments NTA was administered at levels of 0·073, 0·11, 0·365, 0·73, 1·8, 3·65 or 7·3 mmol/kg body weight. Groups of two to seven animals were treated at each dose level and all the rats were killed 6 hr after dosing. Control animals received equivalent volumes of deionized-distilled water.

At the end of a treatment period the rats were anaesthetized with pentobarbital (5 mg/100 g) or ether and the kidneys were quickly excised. After removal, the kidneys were sectioned longitudinally and placed in a fixative solution consisting of 4% formaldehyde and 2% glutaraldehyde in 0·1 M-phosphate buffer, pH 7·3. Sections for light microscopy were prepared by standard techniques and stained with haematoxylin and eosin. Two longitudinal sections were cut from an area near the midline of each kidney and examined by light microscopy at a total magnification of × 125.

The blood and kidney levels of NTA were determined in a series of experiments using ^{14}C-labelled NTA. Nitrilotri[1-^{14}C]acetic acid ([^{14}C]NTA) was purchased from Amersham/Searle Corporation (Arlington Heights, IL). This material was recrystallized and had a purity of 100 ± 2% as determined by reverse isotope dilution analysis. Prior to administration, the pH of the [^{14}C]NTA solution was adjusted to 8·0 using concentrated NaOH. Depending on the dose administered, the concentration of the [^{14}C]NTA solutions was either 0·009, 0·09 or 0·9 mmol/ml. The specific activities of these solutions ranged from 4·0 to 35·0 μCi/ml. Samples of blood and kidney were collected 1, 6, 18 and 24 hr after treatment with a dose of 0·073, 0·73 or 7·3 mmol/kg [^{14}C]NTA.

RESULTS

The development of vacuoles of various sizes in the cytoplasm of proximal tubular epithelial cells was the only treatment-related structural change observed in the kidneys of rats that received NTA. Adjacent parts of the renal tubules, e.g. the distal and collecting tubules, were not affected.

The distribution of the vacuolar changes in the renal cortex was focal. In any given tissue section the entire vacuolated area usually consisted of three or four tubule cross sections surrounded by normal parenchyma (Fig. 1). This pattern was evident even at the highest dose levels of NTA used where it is estimated that detectable cytoplasmic vacuolation was present in less than 2% of the renal proximal tubules. The number of focal lesions observed per animal in the treated rats was relatively constant at all but the lowest dose level of NTA (Table 1). However, the number and size of the vacuoles in the affected tubules varied greatly depending on the dose of $Na_3NTA.H_2O$ administered.

Dose-response experiments

Three grades of cytoplasmic vacuolation were recognized in the proximal-tubule cells following NTA treatment. The most severe vacuolation was observed at the two highest dose levels of NTA used (7·3 and 3·65 mmol/kg). In these rats the affected proximal tubule cells were swollen and their cytoplasm was usually completely displaced by vacuoles (Fig. 1). These vacuoles ranged in size from $<0·2$ μm to 7 μm in diameter. When 1·8 or 0·73 mmol $Na_3NTA.H_2O$/kg was administered to test animals, less swelling of the proximal tubule cells was observed than at the higher dose levels. Vacuoles still filled the cytoplasmic compartment of the affected cells, but they were generally small, only occasionally reaching a maximum diameter of 3·0 μm (Fig. 2). No swelling was observed in the vacuolated proximal tubule cells of rats that received 0·365 or 0·11 mmol/kg doses of NTA. The vacuoles observed were usually 0·5 to 0·75 μm in diameter (Fig. 3), but occasionally attained a diameter of 2·0 μm. In addition, fewer vacuoles were observed per cell than at the higher dose levels of NTA. Usually, three or four vacuoles were visible in each cell, so only a portion ($<50\%$ of the cytoplasm was occupied by vacuoles.

When compared with renal tissues from control animals, no change in tubular cell structure was observed in rats that received a 0·073 mmol/kg dose of NTA. Although some vacuolation was evident in the proximal tubule cells of these NTA-treated rats, it could be distinguished from the NTA-associated vacuolar change by the uniform size of the vacuoles and their position adjacent to the lumen of the tubule (Fig. 4). This type of vacuolation was not recognizably different from that observed in the renal tissue of control animals (Fig. 5).

Table 1. *Incidence of vacuolar lesions in proximal renal tubules of NTA-treated Sprague–Dawley rats*

Dose (mmol NTA/kg)	No. of rats treated	No. of treated rats observed to have focal vacuolar lesions	Total no. of focal vacuolar lesions observed in all kidney sections examined
0 (control)	5	0	0
0·073	3	0	0
0·11	3	1	2
0·365	3	1	4
0·73	3	2	2
1·83	2	1	2
3·65	2	2	3
7·3	7	5	12

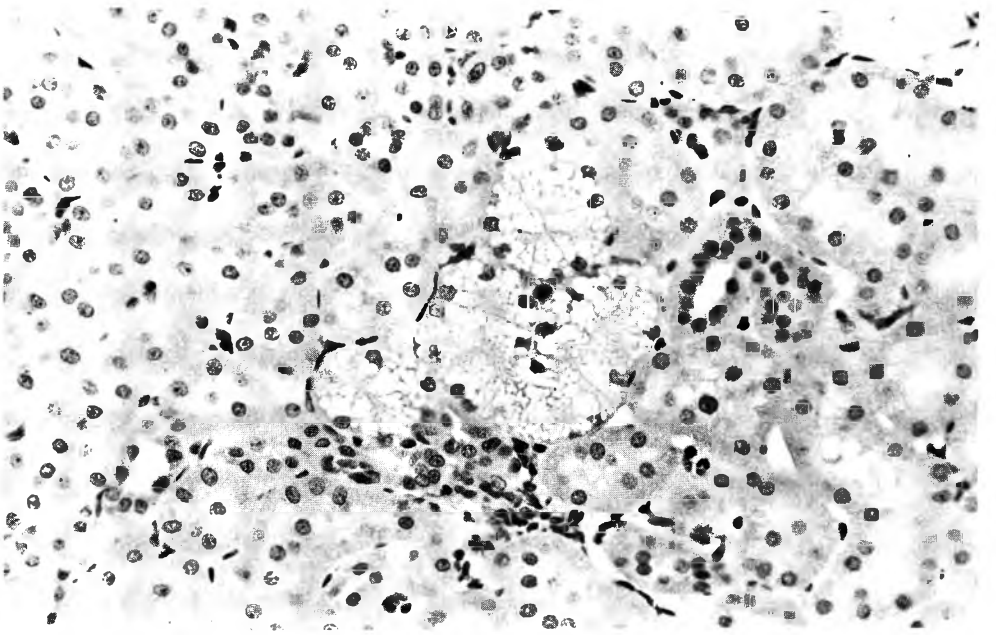

Fig. 1. Focus of cytoplasmic vacuolation in renal proximal tubule cells 6 hr after a dose of 7·3 mmol NTA/kg. Haematoxylin and eosin (H & E) ×265.

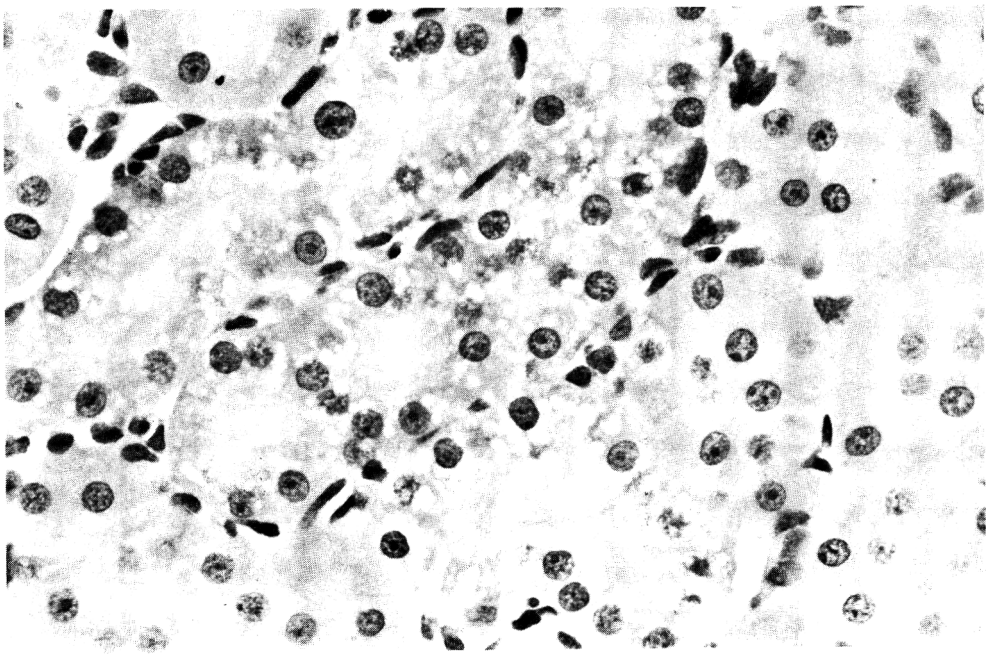

Fig. 2. Proximal tubule cells with an intermediate level of cytoplasmic vacuolation 6 hr after a dose of 1·8 mmol NTA/kg. H & E ×412.

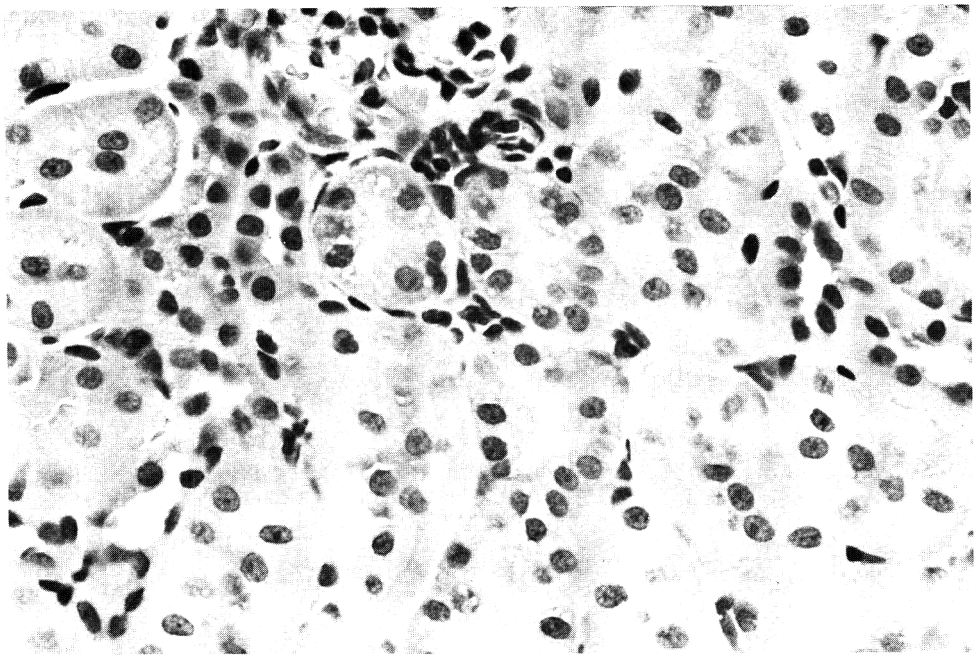

Fig. 3. Very low level of cytoplasmic vacuolation in proximal tubule cells 6 hr after a dose of 0·365 mmol NTA/kg. H & E ×412.

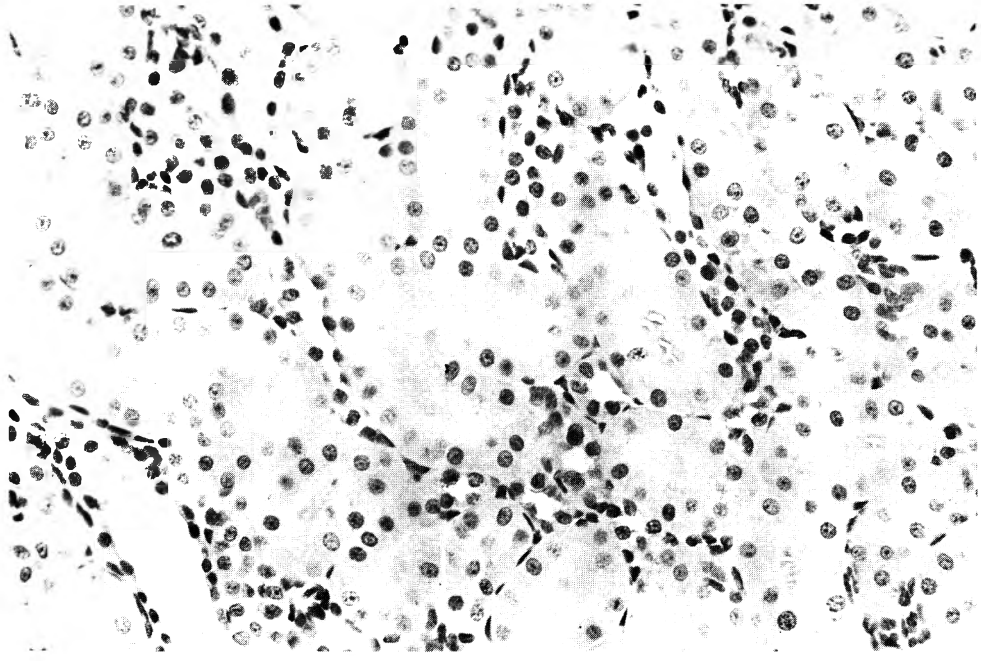

Fig. 4. Small endocytotic vacuoles near the tubule-lumen of the proximal tubule cells 6 hr after a dose of 0·073 mmol NTA/kg. H & E ×265.

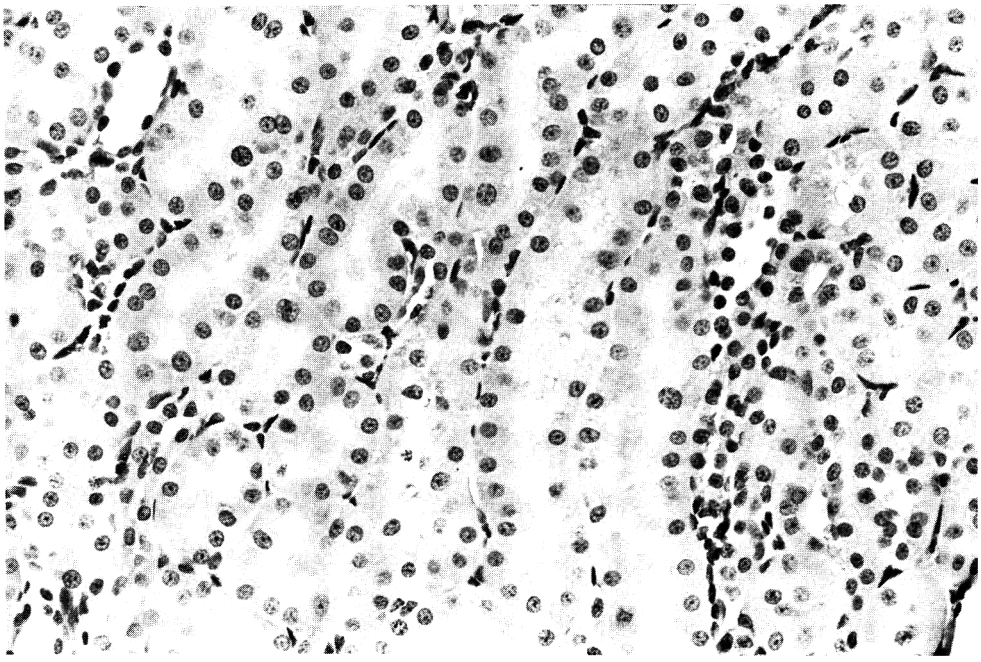

Fig. 5. Endocytotic vacuoles adjacent to the tubule lumen of proximal tubule cells in a control animal. H & E ×265.

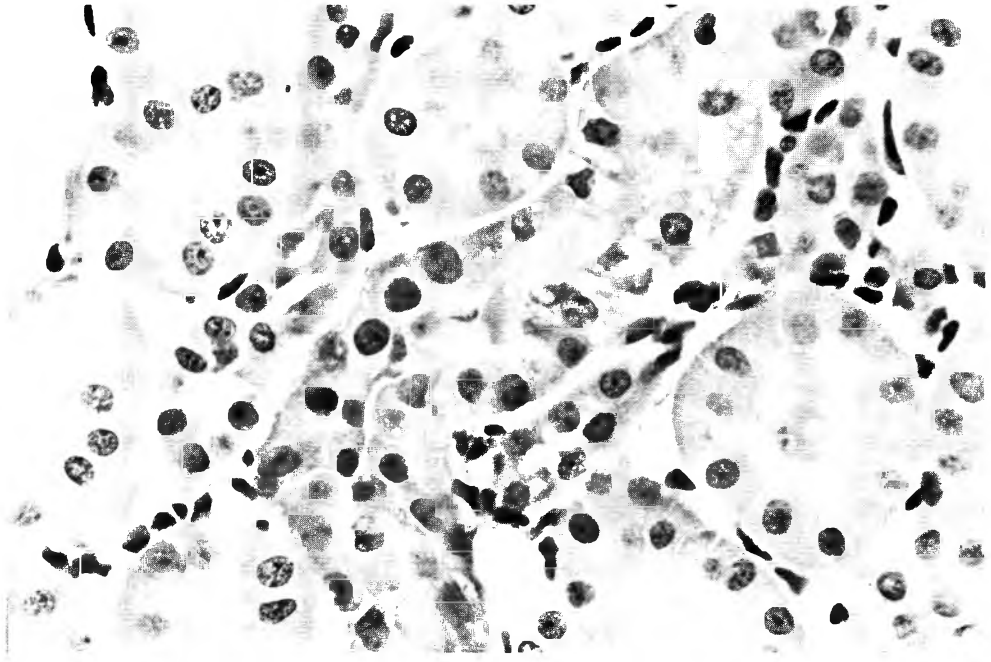

Fig. 6. Mixture of regenerative, vacuolated and non-vacuolated cells in a renal proximal tubule 72 hr after a dose of 7·3 mmol NTA/kg. H & E ×412.

Time-course experiments

No changes were observed in the appearance of renal tissue from rats treated with 7·3 mmol $Na_3NTA.H_2O$/kg 1·5 hr before they were killed. By 6 hr after dosing, however, the formation of multisized vacuoles in the cytoplasm and swelling of the proximal tubule cells was evident. Thereafter these effects of NTA on the proximal tubule cells diminished slowly. By 72 hr after dosing the proximal tubules that were still affected consisted of a combination of regenerative and partially vacuolated epithelium (Fig. 6).

Blood and kidney levels of [^{14}C]NTA

The blood and kidney levels of ^{14}C-NTA are listed in Table 2. The peak levels of ^{14}C-labelled NTA in both blood and kidneys occurred within 1 hr after dosing and had decreased markedly by 18 hr after dosing. The concentration of ^{14}C-labelled NTA was greater in the kidney than in the blood at all the doses and times measured and decreased more slowly in the kidney than in the blood (Table 2). This relatively slow decrease, and the high concentration of NTA in the renal tissue, probably reflect the fact that NTA is cleared from the blood by the kidney and that renal tubular fluid concentrations of NTA are 130 to 200 times greater than those reached in the plasma (Anderson, 1981). Thus a small volume of glomerular ultrafiltrate trapped in the renal tubules could account for most of the observed levels of ^{14}C.

DISCUSSION

The development of cytoplasmic vacuolation in renal proximal tubule cells has been observed following treatment with large doses of sucrose, EDTA and other compounds (Friedman, DeVenuto, Lollini, Mellick & Zuch, 1974; Maunsbach, Madden & Latta, 1962; Schwartz, Hayes, Ide, Johnson & Doolan, 1966; Trump & Janigan, 1962). This vacuolar change was found to develop within a few hours of treatment and to persist for at least 48 hr. Careful analysis of this lesion by biochemical, histochemical, light and electron microscopic techniques has led to the conclusion that the cytoplasmic vacuolation caused by those compounds was due to a perturbation of the endocytotic/lysosomal system of the proximal tubule cells (Maunsbach *et al.* 1962; Schwartz *et al.* 1966; Trump & Janigan, 1962).

In the present study cytoplasmic vacuolation of proximal tubule cells was the only structural alteration observed by light microscopy in renal tissue following oral administration of $Na_3NTA.H_2O$. Comparing previous descriptions of cytoplasmic vacuolation (Friedman *et al.* 1979; Maunsbach *et al.* 1962; Schwartz *et al.* 1966; Trump & Janigan, 1962) with the lesion observed in the present study indicates that the two lesions are similar both in the timing of their development and in their appearance at the cellular level. These similarities suggest that NTA-induced cytoplasmic vacuolation may also be the result of a perturbation of the endocytotic/lysosomal system of the proximal tubule cells. The focal distribution of the NTA-associated lesion, however, contrasts with the diffuse distribution associated with other inducers of cytoplasmic vacuolation. This characteristic difference in response raises the possibility that NTA might be acting on the proximal tubule cells at a site distinct from that affected by either sucrose or other compounds that produce a similar effect. In an attempt to resolve this question a direct comparison of the cytoplasmic vacuolation induced in proximal tubule cells by equimolar doses of sucrose or NTA has been made (Merski, 1980). This work showed that the lesions produced by equimolar doses of these compounds are indistinguishable by light microscopy. In addition, an electron microscopic examination of the NTA-associated response (J. A. Merski, unpublished data, 1980) indicates that NTA does indeed induce vacuole formation through action on the endocytotic/lysosomal system of the renal-tubule cells.

The results of the present study show that the acute effects of NTA on renal tissue are both time- and dose-related. These characteristics are particularly important when considering the chronic urinary tract lesions that develop when test animals receive sufficiently large amounts of NTA (>0·073 mmol/kg/day or *c.* 0·03% of the diet). Since NTA-associated vacuolar lesions may persist for up to 72 hr, daily administration of NTA would not allow a sufficient recovery period for affected tubules. It seems very probable that repeated acute insults, as exemplified by the tubular cell vacuolation, could be involved in the development of the chronic lesions observed in long-term NTA feeding studies. However, if such a relationship between acute and chronic lesion formation

Table 2. *Blood and kidney levels of NTA*

Dose (nmol NTA/kg)	Tissue or fluid	Time after dosing (hr)...	Level of NTA (nmol/g tissue)			
			1	6	18	24
0·073	Left kidney		45·9 ± 9·2	32·1 ± 10·5	6·0 ± 0·5	6·0 ± 1·2
	Right kidney		49·7 ± 3·4	35·0 ± 10·4	6·0 ± 0·6	5·7 ± 0·8
	Blood		5·9 ± 1·0	2·0 ± 0·6	0·4 ± 0	0·4 ± 0·1
0·73	Left kidney		367 ± 129	245 ± 94	46·1 ± 1·7	23·4 ± 4·7
	Right kidney		421 ± 113	276 ± 62	44·8 ± 4·5	21·3 ± 2·1
	Blood		113 ± 8	21·9 ± 4·2	1·6 ± 0·3	0·5 ± 0·2
7·3	Left kidney		1615 ± 164	1639 ± 269	797 ± 306	753 ± 260
	Right kidney		1691 ± 211	1784 ± 281	801 ± 308	771 ± 276
	Blood		279 ± 19·6	132 ± 18	53·7 ± 24·4	54·3 ± 13·3

Values are means ± 1 SD for groups of three animals.

does exist, it would also seem possible that exposure to NTA at levels less than those needed to cause acute lesions (0·073 mmol/kg), would not result in any chronic toxicity. This concept is in agreement with the results of a 2-year feeding study in which no urinary tract lesions were observed in rats fed $Na_3NTA.H_2O$ at a level of 0·03% of their diet (Nixon *et al.* 1972). When adjusted for differences in absorption that occur when NTA is administered by gavage or in the diet (Anderson & Kanerva, 1978; Michael & Wakim, 1971) this dietary level of NTA corresponds almost exactly with the 'no effect' dose observed in the present work (0·073 mmol/kg). Further assessment of the relationship between the acute and chronic NTA-associated urinary tract lesions is currently in progress.

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THE *IN VITRO* ASSESSMENT OF SEVERE EYE IRRITANTS

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Abstract—A method is described for the preliminary screening of new compounds for eye irritancy. Rabbit eyes were enucleated immediately after death and placed in a temperature-controlled chamber where they were superfused with saline solution. Test substances were applied to the eyes and the effects were observed with a slit-lamp biomicroscope. Chemicals reported in the literature to be severely, moderately or non-irritant to eyes were tested using this method, and broad agreement with published observations was achieved. The method provides a means of screening for severe eye irritants without using live animals.

INTRODUCTION

Accidents resulting in eye injury are considered an important industrial hazard and this is reflected in recent United Kingdom legislation (The Protection of Eyes Regulations, 1974; The Protection of Eyes (Amendment) Regulations, 1975). Most industrial injuries are due to mechanical rather than chemical trauma, but chemically induced injuries do occur and the Protection of Eyes Regulations specify that eye protectors are required for "the operation, maintenance, dismantling or demolition of plant..... which contains or has contained acids, alkalis, dangerous corrosive substances which are similarly irritant to eyes.....". This implies that there must be some way of assessing whether or not a new chemical is a "dangerous corrosive substance" or is "irritant to eyes".

A method of assessing whether or not new chemicals are capable of causing serious eye injury is therefore needed. For many years now the method used has involved applying chemicals to the eyes of albino rabbits, using the method described by Draize, Woodard & Calvery (1944). Although this method has been criticized from many angles there is little doubt that it has been, and continues to be useful in identifying severe eye irritants. However, the testing of severely irritant or corrosive materials in this way is likely to cause pain and suffering in experimental animals and this is clearly to be avoided if there are alternatives.

This paper describes an *in vitro* method for screening for severe eye irritants without using live animals.

EXPERIMENTAL

Principle. The eyes are removed from rabbits immediately after death, placed in a temperature-controlled chamber and superfused with isotonic saline. Test substances are applied to the eyes for a set duration (usually 10 sec) then removed by rinsing. The effects are assessed using a slit-lamp biomicroscope.

Animals. New Zealand White rabbits, weighing 1500–2000 g were taken from a stock bred and reared in this laboratory. They were in good health and free from eye defects.

Dissection. Rabbits were killed by an iv overdose of pentobarbitone sodium (Euthatal, May and Baker Ltd, Dagenham, Essex) administered *via* the marginal ear vein. Immediately after death each animal was laid on its side and one drop of isotonic NaCl (Sodium Chloride Injection, BP, 0·9% w/v, The Boots Company, Nottingham) was applied to the surface of the eye to prevent drying during dissection. The nictitating membrane was deflected and the conjunctivae between the nictitating membrane and the eyeball cut with fine scissors. The eyeball was gently proptosed with gentle pressure with the fingers above and below the eyelids. The remaining conjunctivae, extra-orbital muscles and optic nerve were then cut with small enucleating scissors (Holborn Surgical Instrument Co. Ltd, London) and the eyeball was lifted from the orbit by holding the stump of one of the extra-orbital muscles with forceps. Care is needed throughout the dissection to avoid touching the surface of the cornea or cutting the optic nerve too close to the eyeball (which can result in rupture and loss of intra-ocular pressure).

The enucleated eye was mounted in a perspex clamp (Fig. 1) with the cornea positioned vertically, and placed in the superfusion chamber.

Superfusion. Eyes were maintained in a superfusion apparatus (Fig. 2) modified from that described by (Mishima & Kudo (1967). This consists of a perspex chamber with six compartments, each designed to hold one eye. Each compartment is supplied with a stainless steel tube through which isotonic NaCl solution is pumped at a rate of 0·1–0·15 ml/min by means of a peristaltic pump (Type MHRE 7, Watson-Marlow, Falmouth, Cornwall). The perspex clamp holding the eye is positioned so that the NaCl solution drips onto the cornea at the limbus and flows

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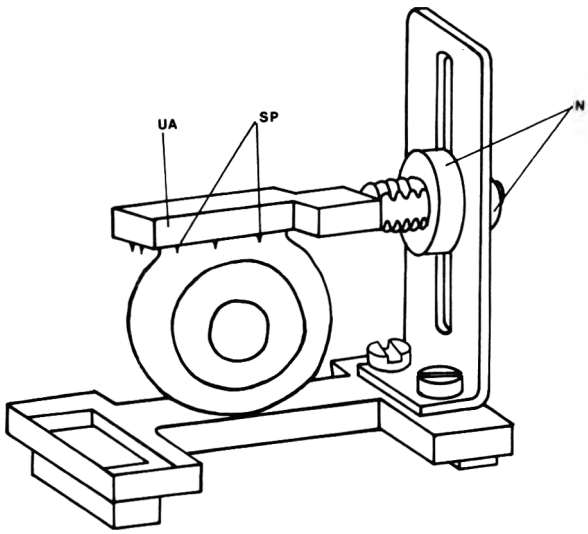

Fig. 1. Perspex clamp for holding enucleated eyes in the superfusion apparatus. The eye is held in place by short lengths of stainless steel pins (SP). These are embedded in the upper arm (UA) of the clamp and protrude for about 1 mm (so that they do not puncture the globe). There is a similar set of pins (not shown) on the base. Part of the upper arm of the clamp is cut away so that saline can drip onto the upper surface of the cornea. The two nuts (N) can be loosened and the upper arm moved up and down to accommodate the eyeball.

down over the corneal surface. The solution is pumped out of the chamber *via* two stainless steel drain tubes at the rear corners. A door at the front of each compartment allows access for observation and manipulation.

The six compartments are enclosed in a water jacket through which water is pumped at approximately 4 litre/min from a temperature-controlled water bath (Circon Unit, Baird and Tatlock, Essex). The temperature of the water bath is adjusted so that the temperature of the compartment and the NaCl drip is 32 ± 1°C, the pre-ocular temperature in the rabbit (Schwartz & Feller, 1962). The NaCl is passed through stainless steel tubes within the water jacket so that it is warmed to the correct temperature before entering the compartments. The temperature of the drip is checked periodically using a thermistor (YSI thermistor, Yellow Spring Co. Inc., OH, USA). The walls of each compartment are made of black perspex which provides a suitable background for slit-lamp observation.

Test chemicals. A range of chemicals was chosen to represent severe, moderate and non-, eye irritants. We did not feel justified in carrying out tests on live animals with these chemicals and therefore relied predominantly on work reported in the literature. The substances tested are listed in Table 1.

Test method. Enucleated eyes were placed in the superfusion chamber and examined (see below) to ensure that they had not been damaged during dissection. They were left in the apparatus for 45–75 min to equilibrate. Test substances were applied to the eye from a 1 ml syringe (2 drops, *c.* 45 μl) so that the cornea was continuously bathed with the test substance for 10 sec. (This period was selected after experiment, to give the best discrimination between irritant and non-irritant substances.) The corneal surface was then rinsed thoroughly with approximately 20 ml of warm isotonic NaCl solution. Solids were tested by applying 50 mg for 10 sec then rinsing as before. The NaCl superfusion was stopped whilst the test material was applied to the eyes and restarted again immediately after they had been rinsed.

All substances, except sodium hydroxide, were tested on four eyes. The effect of different concentrations of sodium hydroxide and of exposing eyes for different periods of time will be described in more detail elsewhere (M. York, R. S. Lawrence & A. B. G. Burton, unpublished data, 1980).

Assessment of effects. Eyes were examined before death using a Haag-Streit Slit Lamp (Clement Clarke, London) and any with abnormalities were rejected. At the same time the corneal thickness was measured using the Depth Measuring Attachment No. 1 for the slit lamp (Burton, 1972; Maurice & Giardini, 1951; Mishima, 1968; Mishima & Hedbys, 1968). Five measurements were taken at the corneal apex and the scale on the depth-measuring device was read to the nearest 0·01 units (*c.* 0·008 mm). The eyes were re-examined after they had been placed in the superfusion apparatus to ensure that they had not been damaged during dissection. On some occasions 2% fluorescein sodium (Smith and Nephew Ltd, Birmingham) was applied to the surface of the cornea for a few seconds and then rinsed off with isotonic NaCl solution. Eyes that were significantly swollen after dissection, that stained with fluorescein or that showed any other signs of damage, were rejected.

The eyes were re-examined at intervals for up to 4 hr after the application of the test substances and any changes in the normal appearance of the cornea were carefully noted. The corneal thickness was also measured and expressed either in terms of instrument units (1 unit ≃ 0·8 mm) or as corneal swelling, which was expressed as [(corneal thickness at time t/corneal thickness before treatment) − 1] × 100%.

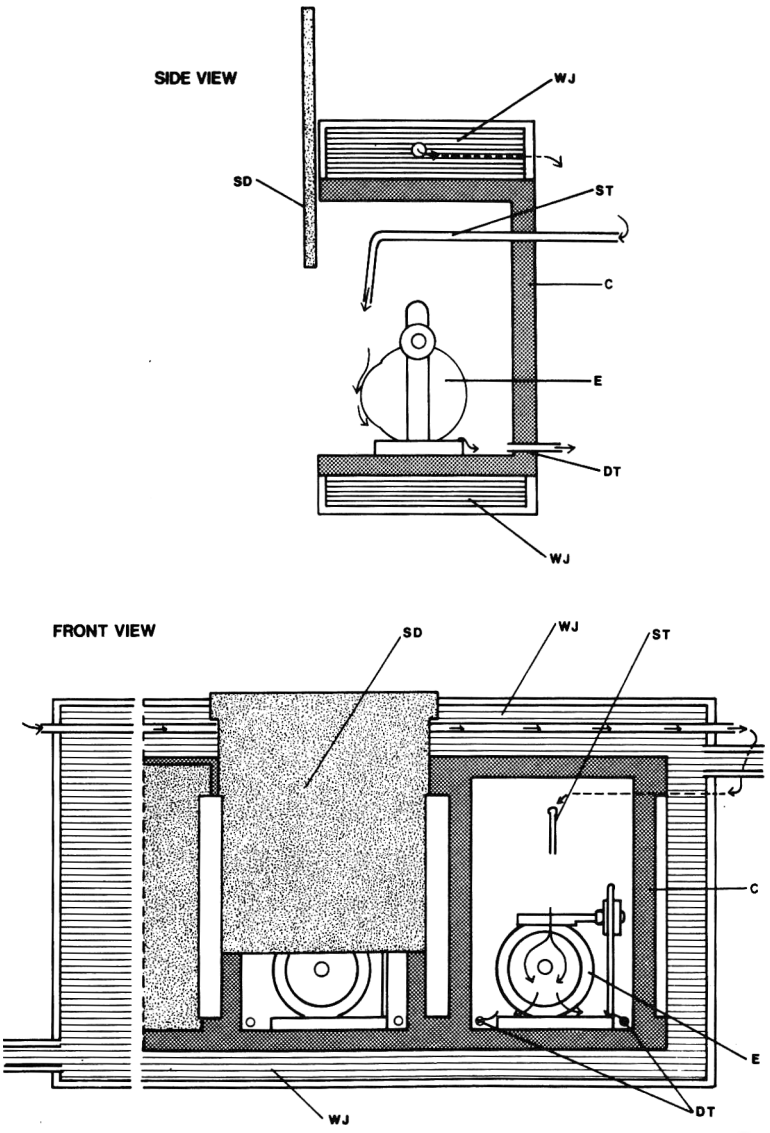

Fig. 2. Schematic view of superfusion apparatus. The enucleated eye (E) in its perspex clamp is placed inside a black perspex compartment (C) surrounded by a water jacket (WJ) through which warmed water is pumped. Saline solution passes *via* stainless steel tubing through the water jacket and then *via* plastic tubing into a stainless steel tube (ST) that enters the back of each compartment. The position of this tube is adjustable so that saline drips over the surface of the eye. Saline is pumped out of the back of the compartments *via* drain tubes (DT). Each chamber has a sliding door (SD).

RESULTS

Viability of eyes kept in the superfusion apparatus

It is important that the corneas of eyes kept in the superfusion apparatus should remain viable for the duration of the experiments. We have shown that this is the case as follows.

Figure 3 is a photomicrograph of a cornea that has been kept in the superfusion apparatus for 4 hr. This shows that the epithelium is intact and has the normal number of layers of cells, and that the stroma is not hydrated. The poor preservation of the endothelium is almost certainly an artifact and endothelia of eyes taken immediately after death and processed in the same manner had a similar appearance.

When eyes were kept in the apparatus and the corneal thickness was measured at intervals over a 4-hr period, there was a slight gradual increase in thickness. The total swelling over the 4-hr period was about 4%.

Figure 4 shows the results of an experiment in which two enucleated eyes were placed in cold (5°C) sodium chloride (0·9%) solution overnight and then placed in the superfusion apparatus at 32°C. Corneal thickness was measured at intervals afterwards. The thickness of one of the two corneas returned to the value measured in the live animal. The reduction in thickness in the second eye was not as great, but there was still a significant reduction in thickness during

Table 1. *Irritancy ratings resulting from* in vitro *eye-irritancy tests and previously published irritancy grades for the same chemicals*

Chemical*	Source†	Irritancy rating‡	Irritancy grade (Carpenter & Smyth, 1946)
Sodium hydroxide (1 N-aqueous)	Hopkin & Williams, Romford, Essex	Severe	10
Acetic anhydride	BDH Chemicals Ltd, Poole, Dorset	Severe	9
Formaldehyde (40% aqueous)	Solmedia Ltd, London	Severe	8
Allyl alcohol	Hopkin & Williams	Moderate/severe	5
n-Butanol	May & Baker, Dagenham, Essex	Moderate	7
Ethanol	James Burrough Ltd, London	Moderate	3
Acetone	Fisons Scientific Apparatus Ltd, Loughborough, Leicester	Slight	5
Cetyl trimethyl ammonium bromide (50 mg solid)	BDH Chemicals Ltd	Slight	Not included
Toluene	Fisons Scientific Apparatus Ltd	Negligible/slight	7
Propylene glycol	Hopkin & Williams	Negligible	1
Glycerine	BP	Negligible	1

*All of the test chemicals were normal laboratory reagents, except glycerine which was to British Pharmacopoeia standard. They were tested undiluted except where otherwise indicated. The results of Carpenter & Smyth (1946) were for undiluted chemicals except where the grade was greater than 5 when various dilutions were used.

†The list of suppliers is relevant to the work reported here but not to that of Carpenter & Smyth (1946).

‡The irritancy rating is somewhat arbitrary, but is based on the following criteria. Severe = Direct effect on the corneal stroma. Moderate = Direct effect on the corneal epithelium. Stromal opacity delayed and probably secondary to effect on epithelium. Swelling in excess of 50% in 4 hr. Slight = Effect on corneal epithelium. Stromal opacity is delayed and is probably secondary to effect on epithelium. Swelling less than 50% in 4 hr. Negligible = No more than slight pitting of epithelium and slight permeability to fluorescein. Swelling less than 10% in 4 hr. The order in which the chemicals are listed gives a further indication of their irritancy ranking as determined by the *in vitro* test results.

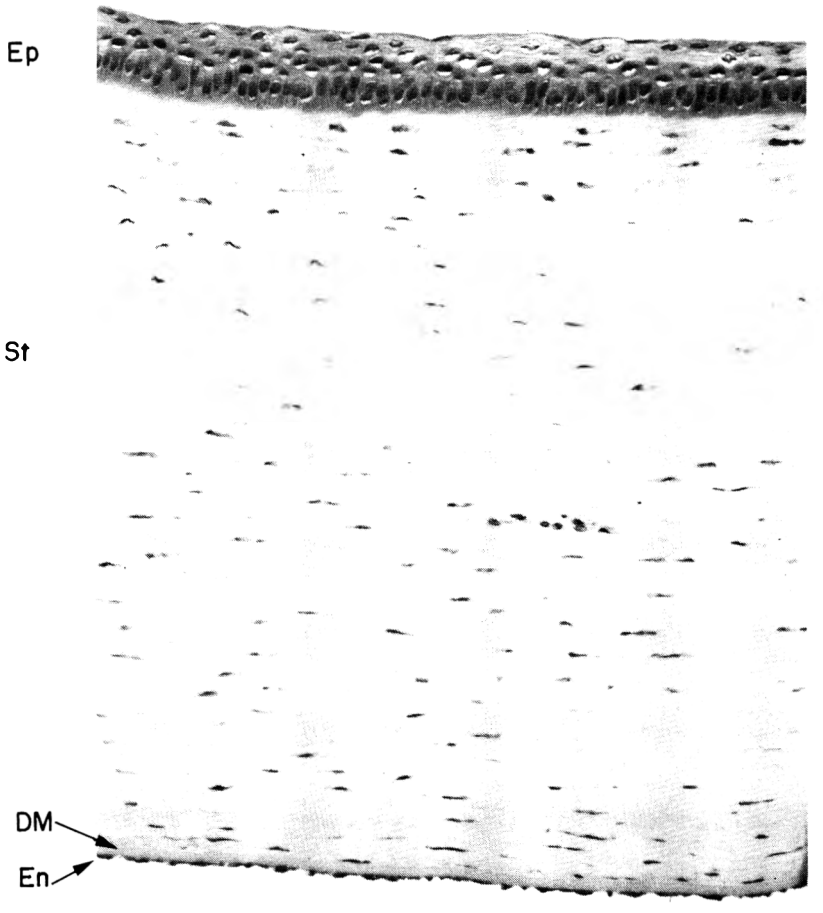

Fig. 3. Section through the cornea of a rabbit eye after 4 hr in the superfusion apparatus. Ep—epithelium, St—stroma, DM—Descemet's Membrane, En—endothelium. Fixed with Davidson's fixative, haematoxylin and eosin × 320 (actual magnification of plate).

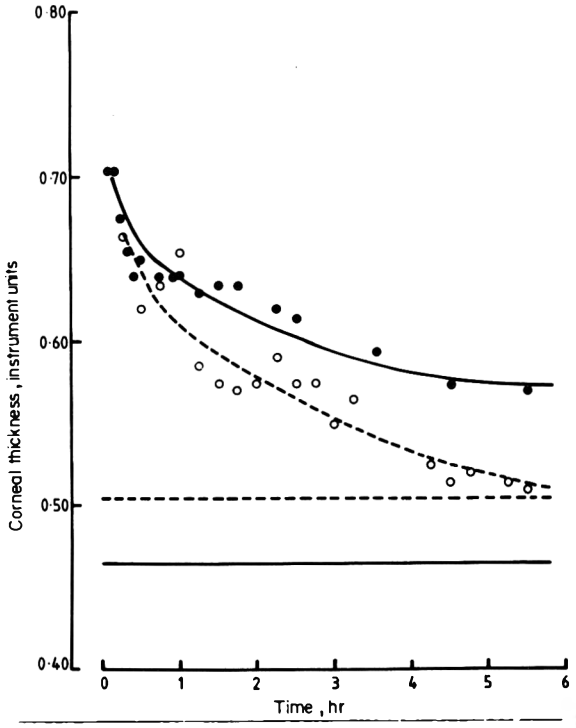

Fig. 4. Temperature reversal effect. The corneal thickness (in depth-measuring-device units, 0·1 unit ≃ 0·08 mm) of two eyes (— and ---) is plotted against time in the superfusion apparatus at 32°C. The eyes were previously immersed in cold (5°C) saline overnight. The horizontal lines (— and ---) indicate the corresponding corneal thicknesses before enucleation. Each point is the mean of five observations.

the course of the experiment. This "temperature reversal effect" is a property of metabolically active endothelia and epithelia (Davson, 1955; Harris, 1960).

Effects of chemicals

Sodium hydroxide. Sodium hydroxide caused rapidly developing and intense opacity of the anterior stroma of the cornea. The areas of opacity had well defined margins. The epithelium remained relatively clear, but was permeable to fluorescein. There was some difficulty in measuring corneal thickness because of the intensity of the stromal opacity, but measurement in areas not affected in this way showed that there was only a modest amount of swelling, thickness increasing by about 50% in 4 hr.

Acetic anhydride. Acetic anhydride caused rapidly developing opacity of both the epithelium and the anterior stroma. In this case the epithelium remained largely impermeable to fluorescein. The areas of opacity had clearly defined borders. Mean corneal swelling after 4 hr was 64 ± 10% (1 SD).

Formaldehyde. Formaldehyde (40%) caused rapidly developing epithelial opacity, slight pitting of the corneal epithelial surface and an increased permeability to fluorescein. A moderately intense stromal opacity was seen soon after application of formaldehyde. Corneal thickness increased after 4 hr by 41 ± 5%.

Allyl alcohol. Allyl alcohol caused rapidly developing opacity of the epithelium and anterior stroma. The epithelium became very permeable to fluorescein and there was very marked stromal oedema. Corneal thickness increased after 4 hr by 87 ± 6%.

n-*Butanol.* *n*-Butanol caused rapidly developing but not very intense opacity of the epithelium, probably because of the loss of some of the surface cells, and the epithelium became very permeable to fluorescein. Some areas of epithelium gradually became detached during the course of the experiment. Stromal opacity and oedema developed gradually. Corneal swelling after 4 hr was 77 ± 10%.

Ethanol. Ethanol caused opacity of the epithelium and increased permeability to fluorescein. Areas of the epithelium became detached from the cornea. Stromal opacity and oedema developed gradually. The increase in corneal thickness after 4 hr was 65 ± 7%.

Acetone. Acetone caused slight opacity of the epithelium, probably representing the loss of some of the surface layers of cells, and increased permeability to fluorescein. There was little stromal opacity. Corneal thickness increased after 4 hr by 28 ± 7%.

Cetyl trimethyl ammonium bromide. Solid cetyl trimethyl ammonium bromide caused slight opacity of the corneal epithelium. Epithelial permeability to fluorescein increased markedly in small well-defined areas. There was little stromal opacity. Corneal thickness increased after 4 hr by 27 ± 2%.

Toluene. Toluene caused relatively little effect in the experiments. The epithelium became slightly pitted and there was an increased permeability to fluorescein. There was little stromal opacity. Corneal thickness increased after 4 hr by 13 ± 5%.

Propylene glycol. Propylene glycol caused a very slight increase in fluorescein permeability, but no opacity. Corneal thickness increased after 4 hr by 10 ± 5%.

Glycerine. Glycerine was virtually without effect in these experiments. Corneal thickness increased after 4 hr by 5 ± 2% which is comparable with the change in thickness in untreated eyes over the same period.

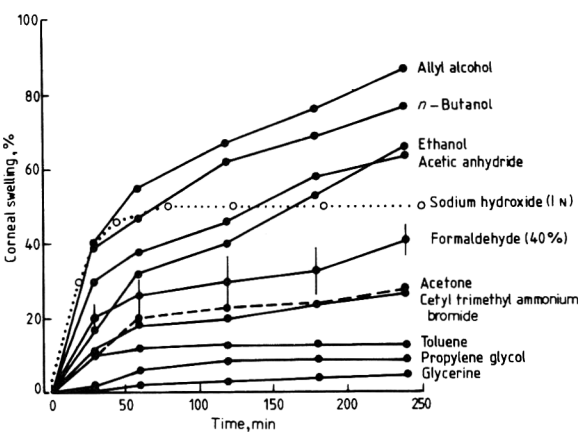

Fig. 5. Corneal swelling following insult with ten chemicals of differing irritancy. Each point represents the mean swelling of four eyes treated with each chemical except in the case of sodium hydroxide, for which the swelling of only one eye is presented (O). Points for acetone are joined by a dashed line for clarity only. Vertical bars represent 2SD. For the sake of clarity this is only shown for swelling following treatment with formaldehyde; other points have comparable standard deviations.

The changes in corneal thickness following the application of these chemicals are summarized in Fig. 5.

DISCUSSION

The corneas of eyes maintained in the superfusion apparatus remain in good condition for the duration of the experiments. Structural changes are not apparent by light microscopy and the cornea remains metabolically active, as shown by the temperature reversal experiments. There is however a slight increase in thickness of the cornea, amounting to about 4% of the initial thickness, during a 4-hr period. Previous, unpublished, experiments have shown that the swelling of the cornea in the superfusion apparatus is not reduced significantly by using fluids with an ionic composition similar to that of tears, but some improvement can be achieved by regulating the intra-ocular pressure. This may be done by cannulation through the optic nerve. However, for the purposes of these experiments, in which rather gross changes in appearance of the cornea were sought, these sophistications were not used, but eyes with an obvious loss in intra-ocular pressure were not used.

The effects of these chemicals *in vitro* fall broadly into three categories. Sodium hydroxide and acetic anhydride cause very rapid and intense opacity of the epithelium and stroma. The solvents, *n*-butanol, toluene, acetone and ethanol affect mainly the corneal epithelium, causing loss of surface cells, an increase in permeability to fluorescein (and therefore water) and a consequent oedema and opacity of the corneal stroma. The same effect was caused by the detergent, cetyl trimethyl ammonium bromide. Finally propylene glycol and glycerine had little effect.

Two chemicals, formaldehyde and allyl alcohol have effects which do not fall clearly into these categories. Formaldehyde seemed to affect both the epithelium and the stroma directly, although stromal opacity did not develop immediately. The degree of stromal opacity was greater than would normally be expected from the degree of swelling. Formaldehyde, therefore, seems to fall into the same category as sodium hydroxide and acetic anhydride in affecting the stroma directly. Allyl alcohol, on the other hand, has a rapid effect both on the epithelium and on the stroma, but the development of swelling and opacity was fairly typical of the other solvents.

The direct effect of sodium hydroxide, acetic anhydride and, probably, formaldehyde on the stroma is likely to be of more serious consequence than the secondary swelling caused by the solvents because the prognosis for eventual recovery is less favourable. On this basis the order of irritancy shown in Table 1 was derived. The criteria used to distinguish between severe, moderate, slight and negligible irritants are also given. Our irritancy ranking can be compared with the irritancy grades of Carpenter & Smyth (1946) which are also listed in Table 1. There is good agreement between the two schemes as far as the three most irritant chemicals are concerned and for the two least irritant, but the correlation is poor for the solvents. In particular ethanol and allyl alcohol showed greater relative irritancies in the *in vitro* test compared with the findings of Carpenter & Smyth (1946), while toluene and acetone had less effect *in vitro*. However Carpenter & Smyth arrived at their grading of irritancy by considering the concentration, or amount, required to produce an effect of given severity rather than by considering the relative severity of the response produced by a given insult as we did. The order of irritancy resulting from the *in vitro* experiments is further supported by a more detailed comparison of the effects seen *in vitro* with those reported in the literature both for experimental animals and for man.

Sodium hydroxide. Caustic solutions of sodium hydroxide applied to the eyes of experimental animals cause destruction of the corneal epithelium, dense stromal opacification and destruction of the endothelium after contact for 1 min. Ulceration of the cornea frequently follows with eventual perforation and complete loss of the eye (Green, Sullivan, Hehir, Scharpf & Dickinson, 1978; Matsuda & Smelser, 1973; Vrabec & Obenberger, 1976a,b; Vrabec, Obenberger & Vrabrec, 1975). We did not find that the epithelium was lost *in vitro*, but there was a considerable increase in permeability and light and electron micrographs showed a considerable loss of cellular structure (M. York, R. S. Lawrence and A. B. G. Burton, unpublished data, 1980). In man, caustic alkali burns are a significant clinical problem and can frequently lead to loss of sight even after brief contact (see for example Grant, 1974).

Acetic anhydride. There does not seem to be a detailed description of the effect of acetic anhydride on rabbit eyes, but Carpenter & Smyth (1946) included it in their grade 9 for irritancy. Only sodium hydroxide and maleic anhydride were more irritant. Acetic anhydride has caused loss of vision in man, although slow recovery is a more usual outcome of accidental contact (McLaughlin, 1946; Grant, 1974). There is therefore good agreement between the findings *in vitro* and published reports of the irritancy of acetic anhydride.

Formaldehyde. Formaldehyde has a delayed effect on the cornea in both rabbit and man. Fixation of the stroma is said to reduce corneal swelling (Grant, 1974), which agrees very well with our observations. Permanent loss of vision has been reported in man following accidental contact, but gradual recovery of sight is more usual (Grant, 1974).

Allyl alcohol. Allyl alcohol caused only moderate lesions in rabbit eyes, with some corneal opacity and "corneal slough", presumably loss of epithelium; recovery was within one week (Dunlap, Kodama, Wellington, Anderson & Hine, 1958). However it has also caused moderately severe corneal opacities in man and, under rare circumstances, loss of vision (Grant, 1974). The response to allyl alcohol *in vitro* therefore seems to be greater than that reported to occur in the live rabbit. However the fact that eye damage has been seen in man suggests that the *in vitro* test may give a valid indication of irritant potential.

n-Butanol. Carpenter & Smyth (1946) include this amongst their grade 7 irritants, and it has been found that solutions of butanol readily loosen the epithelium from excised beef cornea (Hermann & Hickman 1948). Butanol does not seem to cause human eye injury, although the vapour causes a superficial, vacuolar keratitis (Grant, 1974). Our *in vitro* results

are consistent with published accounts of butanol irritancy in experimental animals.

Ethanol. Carpenter & Smyth (1946) included ethanol among their grade 3 irritants, the least irritant of the solvents we examined, but *in vitro* work showed a significant effect. This is supported by work at this laboratory in which 95% ethanol was applied to one eye of each of two live rabbits. This caused loss of the corneal epithelium and a gradual swelling of the corneal stroma. The rate of swelling was less than that seen *in vitro* and the maximum swelling achieved was only 50%, compared with the 64% *in vitro*. Ethanol causes injury of the corneal epithelium in man and, exceptionally, corneal opacity (Grant, 1974).

Acetone. Carpenter & Smyth (1946) classed acetone as a grade 5 irritant, but it had relatively little effect *in vitro*. No work has been done on this solvent in this laboratory, but epithelial damage and some corneal opacity have been reported in experimental animals (Grant, 1974). In man acetone has caused only corneal epithelial damage, with the one exception of a worker who splashed a mixture of acetone and cellulose acetate into his eye. The cellulose acetate formed a cast on the eye and it is believed that this prolonged the contact with acetone; deep corneal injury resulted (Grant, 1974).

Cetyl trimethyl ammonium bromide. Grant (1974) reported marked corneal opacity in rabbits and man following the introduction of an ointment containing 15% of this detergent into the anterior chamber. In this laboratory dilute solutions of this detergent and other similar cationic detergents, have been applied to rabbit eyes and these solutions have been found to be moderately, sometimes severely, irritant. The relatively slight effect of cetyl trimethyl ammonium bromide *in vitro* was therefore, at first, rather surprising. However the detergent was applied *in vitro* as a solid, an experiment that we have not done on live animals, and it is probable that, during the 10 sec contact time, only a small amount dissolved. Other detergents that have been tested *in vitro* have caused loss of corneal epithelium and consequent swelling of the stroma, an observation that has also been made on live animals.

Toluene. Carpenter & Smyth (1946) included toluene amongst their grade 7 irritants, but other workers have found only slight irritation (Grant, 1974). In this laboratory application of 5 μl of toluene to rabbit eyes caused only slight irritation, but 100 μl of a 15% solution of toluene in polyoxyethylene sorbitan mono-oleate (Tween 80) was moderately irritant to eyes, probably because the rather viscous Tween solution maintained contact with the cornea for a prolonged period. Grant (1974) suggested that the irritancy probably depends on the contact time. This is supported by our work on live animals and by the *in vitro* findings.

Propylene glycol and glycerine. These two chemicals had little effect *in vitro* and were in the least irritant group of the classification of Carpenter & Smyth (1946). They have not caused eye injury in man (Grant, 1974).

The results of using the *in vitro* test method described in this paper, with a variety of chemicals, correlate broadly with *in vivo* observations made in this laboratory and others described in the literature. The three most irritant substances in the literature were also found to have the greatest effect *in vitro* and the effects seen were consistent with those described previously. The two substances that were the least irritant *in vitro* are generally agreed not to be eye irritants. There were, however, apparent discrepancies between the relative irritancy of a series of solvents *in vitro* and published accounts of animal experiments. However it is not clear whether these are real discrepancies and, in some instances at least, the *in vitro* results seem to reflect the hazard to man. Perhaps the best way to resolve this issue would be to mount a more systematic comparison of effects on live animals and the *in vitro* system, but we did not feel justified in doing this.

It is not claimed that this method provides a complete alternative to the testing of chemicals on live animals. Amongst other deficiencies of the method, it clearly takes no account of recovery from insult, which is an important criterion for assessing potential hazard, nor of possible effects on the conjunctivae. However this method provides a means of identifying chemicals that are severely or moderately irritant to eyes without using live animals. Live animal experiments may still be necessary before a new chemical is used by man but they should not be carried out before the possibility of severe irritation has been excluded using an *in vitro* test such as that described above, or a comparable *in vitro* procedure.

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SHORT PAPERS

DETECTION OF NITROSAMINES IN A COMMONLY USED CHEWING TOBACCO

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Abstract—Analysis of a popular brand of Indian chewing tobacco (Pandharpuri) for *N*-nitroso compounds was carried out using high-pressure liquid chromatography and thin-layer chromatography techniques. It was demonstrated that this variety of tobacco contained nitrosodimethylamine, nitrosopyrrolidine and nitrosohydroxyproline, each at levels of about 1–2 μg/g tobacco, as well as some nitrosonornicotine. Fractions containing the nitrosamines were subsequently subjected to analysis by gas–liquid chromatography using a thermal energy analyser, to confirm the other findings and determine the concentrations involved. The results obtained, the large numbers of tobacco chewers in India, and the ability of these nitrosamines to induce tumours in animals point to the importance of studying nitrosamine levels in the saliva of tobacco chewers and of non-chewers.

Introduction

Epidemiological studies have demonstrated an association between tobacco chewing and cancer of the oral cavity and oesophagus (Moore, Bissinger & Prochl, 1953; Wynder & Bross, 1961; Wynder, Bross & Feldman, 1957). The chewing of betel quids, particularly those containing tobacco, correlates with high incidence rates of cancer of the upper digestive tract in India and other countries in the Orient (Khanolkar, 1950; Sanghvi, Jayant & Pakhale, 1980).

In experimental animals, tobacco extract and tobacco smoke have been shown to be tumorigenic (Bock, Shamberger & Myers, 1965; Dontenwill, Chevalier, Harke, Lafrenz, Reckzeh & Schneider, 1973; Wynder & Hoffman, 1967). Apart from traces of hydrazines, *N*-nitrosonornicotine (NNN) is the only organic carcinogen that has been identified in chewing tobacco (Hecht, Ornaf & Hoffman, 1975a,b). The NNN levels of 3·5–90·6 ppm detected by Fine (1978) in American chewing tobacco are among the highest demonstrated for an environmental nitrosamine in terms of occurrence and human exposure.

Therefore, it was considered to be of interest to study the possible nitrosamine content of a commonly used variety of Indian tobacco known as Pandharpuri.

Experimental

Standard nitroso compounds. *N*-Nitrosoproline, *N*-nitrosohydroxyproline (NHPRO) and *N*-nitrososarcosine were prepared by nitrosation of the corresponding amino acid with sodium nitrite in the presence of HCl. *N*-Nitrosodimethylamine (NDMA), *N*-nitrosopyrrolidine (NPYR) and NNN were prepared similarly by nitrosation of the corresponding secondary amine.

Preparation and use of tobacco extracts. A crude alcoholic extract of chewing tobacco of the Pandharpuri variety was prepared for the detection of nitroso compounds by shaking 50 g tobacco with ethanol at 0°C on an automatic shaker for 2 hr. The extract was kept (tightly corked) at −20°C overnight, and was then filtered. The filtrate was lyophilized to a dry residue, which was analysed by high-pressure liquid chromatography (HPLC).

For confirmation of the nitroso compounds detected, extracts were prepared by shaking 20 g tobacco magnetically overnight in 200 ml acetone–water (2:1, v/v) at 20°C in a flask covered with black paper. Most of the acetone was removed by flash evaporation at 40°C and the aqueous layer was adjusted to pH 10 with NaOH and extracted three times with ethyl acetate, the extracts being combined to give extract EtOAc-I. The remaining aqueous layer was then acidified to pH 2–3 with dilute HCl and extracted exhaustively with ethyl acetate (extract EtOAc-II). Both organic extracts were dried over Na_2SO_4 and the solvents were removed by rotary evaporation at <40°C. The residues were used for studies involving HPLC, preparative thin-layer chromatography (TLC) and gas chromatography using a thermal energy analyser (GLC–TEA), for the confirmation and estimation of the nitrosamines present.

Analytical methods. TLC of the nitrosamines was carried out on silica-gel plates using the procedure of

Vasundhara, Jayaraman & Parihar (1975), with chloroform–methylene chloride (9:1, v/v) as the eluant. For TLC of NHPRO, the procedure of Hansen, Iwaoka & Archer (1974) was used, with elution of the silica-gel plates with 95% ethanol–benzene–water (4:1:1, by vol.). Spots were detected under UV light at 254 nm.

HPLC was carried out on a Model ALC/GPC/244 liquid chromatograph (Waters Associates, Inc., Milford, MA, USA) fitted with a 6000A solvent-delivery system and a 30 cm × 3·9 mm ID μBondapak C_{18} column (Waters Associates). The detector was a Model 440 absorbance detector (Waters Associates) set at 254 nm, and samples were injected via a model U6K injection system. The column was eluted with 0·4% aqueous acetic acid, at a flow rate of either 1·0 or 2·0 ml/min. Chart speed was maintained at 5 mm/min. When necessary, samples were filtered through a Millipore filter (type HA) of 0·45 μ pore size, held in a stainless swinny fitting.

The GLC–TEA analyses were carried out at the Institute of Toxicology and Chemotherapy, German Cancer Research Centre, Heidelberg, FRG, using the procedure described by Spiegelhalder, Eisenbrand & Preussmann (1979).

Quantitative estimations of nitroso compounds. The levels present in tobacco were estimated by comparison of the HPLC peak areas for the test sample with those for known concentrations of the appropriate standards, and/or by GLC–TEA.

Results and Discussion

Comparison of HPLC retention times with those of the standard compounds demonstrated the presence of NDMA, NPYR and NNN as well as of one nitrsoamino acid (NHPRO) in the crude alcoholic extract of tobacco. The elution of NNN required a flow rate of 2·0 ml/min. The HPLC profile of the crude extract eluted at a flow rate of 1·0 ml/min is shown in Fig. 1, showing the separation of NDMA, NPYR and NHPRO. HPLC of the ethyl acetate fractions showed the presence of NPYR, NNN and NDMA in EtOAc-I and of NHPRO in EtOAc-II. These two extracts were then subjected to preparative TLC; the bands corresponding to NHPRO, NDMA, NPYR and NNN were scraped off and eluted with methylene chloride (for the nitrosamines) or with water (for NHPRO) and the eluted bands were checked by HPLC (Figs 2 & 3), the identities being confirmed by comparison of the retention times with those of standard compounds. Figures 2a–c show the presence of NDMA, NPYR and NNN in fractions 3, 8 and 11 derived by

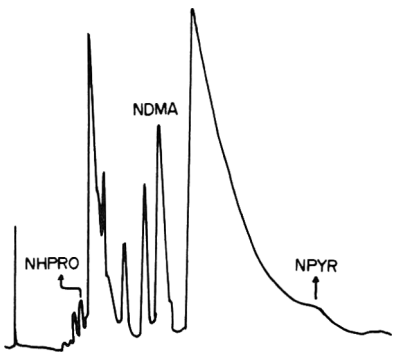

Fig. 1. HPLC profile of the crude alcoholic extract of Pandharpuri tobacco chromatographed on a μBondapak C_{18} column eluted with 0·4% aqueous acetic acid at a flow rate of 1·0 ml/min (chart speed 5 mm/min; detector at 254 nm), showing separation of *N*-nitrosohydroxyproline (NHPRO), *N*-nitrosodimethylamine (NDMA) and *N*-nitrosopyrrolidine (NPYR).

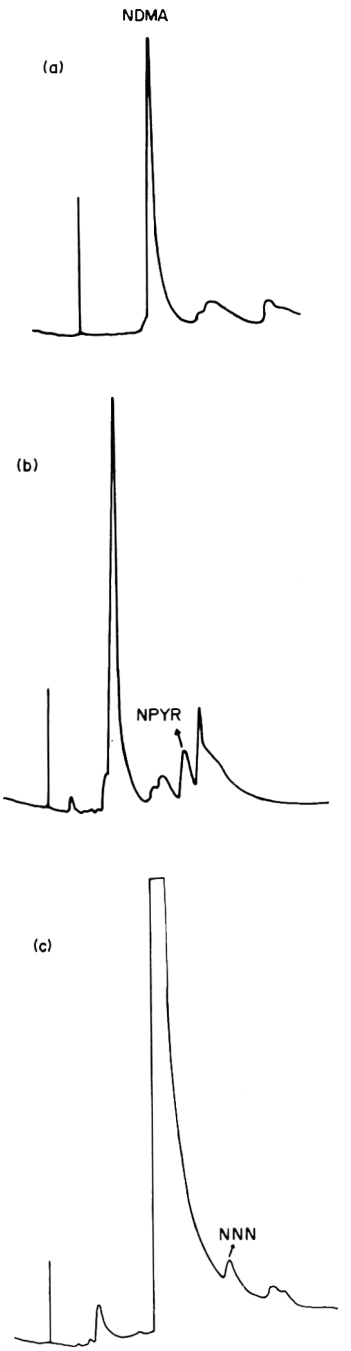

Fig. 2. HPLC profiles of (a) fraction 3—*N*-nitrosodimethylamine, (b) fraction 8—*N*-nitrosopyrrolidine and (c) fraction 11—*N*-nitrosonornicotine, obtained by preparative TLC of ethyl acetate fraction EtOAc-I. The HPLC was carried out on a μBondapak C_{18} column eluted with 0·4% aqueous acetic acid at a flow rate of 2·0 ml/min.

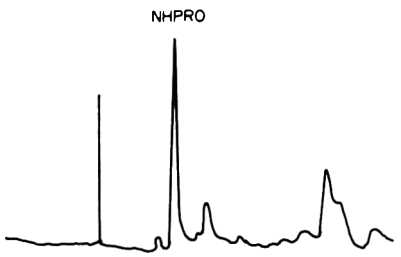

Fig. 3. HPLC profile of *N*-nitrosohydroxyproline in a fraction isolated by preparative TLC of ethyl acetate fraction EtOAc-II and chromatographed on a μBondapak C_{18} column eluted with 0·4% aqueous acetic acid at a flow rate of 1·0 ml/min.

preparative TLC from EtOAc-I, while the presence of NHPRO in a fraction from EtOAc-II is shown in Fig. 3.

HPLC analyses indicated that NDMA was present in the tobacco to the extent of about 2 μg/g and NHPRO at 1 μg/g. It was not possible to calculate the concentration of NPYR as it was marked by other interfering bands. The concentration of NNN was too low to be calculated. GLC–TEA analysis, a sensitive detector for nitrosamines, confirmed the presence of NDMA, NPYR and NNN, at levels of 2–3, 1–2 and 1 μg/g tobacco respectively.

These observations clearly show that the Pandharpuri variety of Indian tobacco contains three nitrosamines (NDMA, NPYR and NNN) and a nitrosoamino acid (NHPRO). All these nitrosamines have been shown to be carcinogenic in laboratory animals (Hecht, Chen & Hoffmann, 1978; Hecht *et al.* 1975a,b; Hoffman, Adams, Brunnemann & Hecht, 1979; Hoffmann, Hecht, Ornaf & Wynder, 1974; Hoffmann, Raineri, Hecht, Maronpot & Wynder, 1975; Lijinsky & Taylor, 1976; Magee & Barnes, 1956; Singer & Taylor, 1976). The presence of nitrosamines in the chewing tobacco acquires significance because vast numbers of people in India are addicted to the habit of chewing it almost continuously, so that their buccal mucosa is in contact with these highly carcinogenic compounds for a long time. Of course, this may not be the only factor or even one of the factors responsible for oral cancer. Many other factors may either contribute to or reduce the nitrosamine pool in the body. Yet, given the presence of detectable quantities of nitrosamines in chewing tobacco, it will be worthwhile to look for these compounds in the saliva of habitual tobacco chewers as well as in the saliva of non-chewers. Work along these lines is in progress.

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CHROMATOPOLAROGRAPHY OF *N*-NITROSAMINES INCLUDING DETERMINATION OF *N*-NITROSO-DIETHANOLAMINE IN COSMETIC PRODUCTS*

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Abstract—A simple and sensitive method for trace level determination of different classes of *N*-nitrosamines is described. The method is based on a combination of high-performance liquid chromatography and differential pulse polarography. The separation of *N*-nitrosamines from their composite mixtures or interfering species is carried out on a reverse phase column followed by continuous detection with the polarographic detector. Analysis at nanogram levels is demonstrated. In addition, procedures are presented for the recovery and determination of *N*-nitrosodiethanolamine in various cosmetic products.

Introduction

N-Nitrosamines constitute one of the most important classes of chemical carcinogens (Magee & Barnes, 1967). Hence considerable effort has been directed towards the development of accurate and sensitive methods for the determination of these compounds in various matrices. Such methods are based upon a variety of techniques and include chromatography (Cox, 1973; Daiber & Preussman, 1964; Klimisch & Ambrosias, 1976), spectroscopy (Daiber & Preussman, 1964; Fine, Lieb & Rufeh, 1975; Fine, Rufeh, Lieb & Rounbehler, 1975; Saxby, 1972) and electrochemistry (Chang & Harrington, 1975; Hasebe & Osteryoung, 1975).

In a recent paper (Vohra & Harrington, 1980) we presented the results of an evaluation of a polarographic detector for high-performance liquid chromatography (HPLC) for the determination of *N*-nitrosamines, using *N*-nitrosodipropylamine as a model compound. To demonstrate the versatility of the technique we have extended the study to the analysis of other types of *N*-nitroso compounds as well as to the determination of *N*-nitrosodiethanolamine (NDELA) in cosmetic products.

Experimental

The instrumental and chromatographic conditions were as previously described (Vohra & Harrington, 1980) except as noted. The cosmetic products were purchased over the counter in the Princeton, NJ area. NDELA was synthesized by the method of Preussmann (1962). The spectral and other physical properties of the isolated compound were the same as those reported earlier by Fan *et al.* (Fan, Goff, Song, Fine, Arsenault & Biemann, 1977). The *N*-nitroso derivative of *N*-methylaniline was prepared by reacting the amine with nitrite under acidic conditions (Vogel, 1957). *N*-Nitrosodiphenylamine was purchased from Aldrich Chemical Company (Milwaukee, WI) and was recrystallized three times from methanol by the addition of water to the cloud point. Methanol-free chloroform was obtained from Waters Associates, Inc., Milford, MA. All other reagents were as reported earlier (Vohra & Harrington, 1980).

For each class of *N*-nitrosamines defined by Fan, Krull, Ross, Wolf & Fine (1978)—for further details see results section—different mobile phases were used for HPLC. These were as follows: class I, methanol–water–glacial acetic acid (50:47:3, by vol.); class II, methanol–water–glacial acetic acid (41:56:3, by vol.); class III, water–glacial acetic acid (98:2, v/v); class IV, water–glacial acetic acid (98:5, v/v). The chromatopolarographic conditions for *N*-nitrosoproline, *N*-nitroso-*N*-methylaniline and *N*-nitrosodiphenylamine were as follows: mode, differential pulse; modulation amplitude, 100 mv; drop size, large; drop time, 0·5 sec; potential −0·736 *v.* Ag/AgCl (*N*-nitrosoproline), −0·740 *v.* Ag/AgCl (*N*-nitroso-*N*-methylaniline and *N*-nitrosophenylamine); flow rate, 2 ml/min. The chromatopolarographic conditions for NDELA are described below and those for *N*-nitrosodipropylamine were as described previously (Vohra & Harrington, 1980).

The procedure for the extraction of NDELA from cosmetic products was as follows. To 6 g of the cosmetic product in a beaker were added 250 mg NaCl, 100 mg ammonium sulphamate and 12 ml of triple distilled and deionized water. The NaCl was added to break up emulsions and the addition of ammonium

*Presented in part at the thirty-first Pittsburgh Conference, Atlantic City, NJ, March 1980.

sulphamate prevented the formation of NDELA during analysis. The contents of the beaker were stirred for 5 min and then extracted with alcohol-free chloroform (3 × 45 ml). The chloroform extracts were discarded and the aqueous extracts were transferred to a centrifuge tube. The beaker was washed twice with 1–2 ml of water and the washings were also transferred to the centrifuge tube. The solution was centrifuged at 25,000 rpm for about 15 min. The supernatant was transferred to a volumetric flask and the volume was adjusted to 25 ml. Five millilitres of the solution, followed by 3 ml water, were passed through an activated C_{18} Sep-Pak cartridge (Waters Associates, Inc.). The first 0·5 ml of eluent coming out of the Sep-Pak was discarded and the rest of the eluent was collected and analysed by chromatopolarography under the following conditions: flow rate, 1·5 ml/min; mode, differential pulse; modulation amplitude, 50 mv; potential, −0·820 *v.* Ag/AgCl; drop size, large; drop time, 0·5 sec. Using these conditions, the retention time of NDELA is 3 min 25 sec.

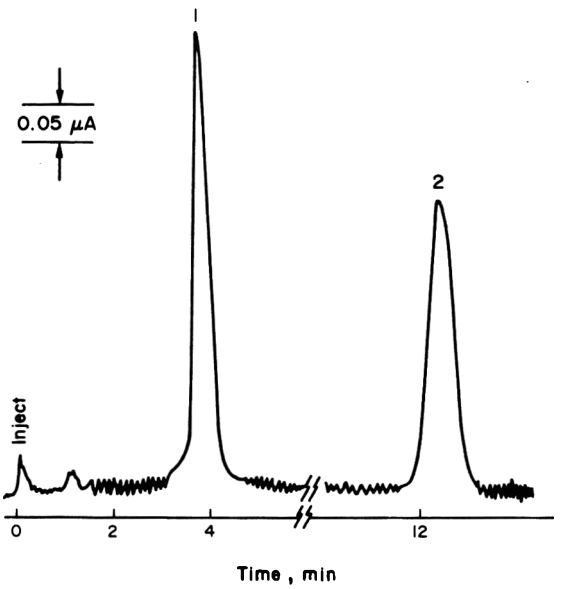

Fig. 2. Chromatopolarogram of (1) *N*-nitroso-*N*-methylaniline (90 ng) and (2) *N*-nitrosodiphenylamine (150 ng). For details of chromatopolarographic conditions, see text.

Results and discussion

For the purposes of separation and analysis, Fan *et al.* (1978) classified *N*-nitroso compounds into four broad and somewhat overlapping classes. The classification is based upon various physical properties such as volatility, ionic character and polarity. The four classes are as follows: (I) volatile *N*-nitroso compounds such as *N*-nitrosodiethylamine, *N*-nitrosodimethylamine and *N*-nitrosodipropylamine; (II) low polarity, non-volatile *N*-nitroso compounds such as *N*-nitrosodiphenylamine and *N*-nitroso-*N*-methylaniline; (III) high polarity, non-ionic, non-volatile *N*-nitroso compounds such as NDELA; (IV) high polarity, ionic, non-volatile *N*-nitroso compounds such as *N*-nitrosoproline, *N*-nitrososarcosine and other *N*-nitrosoamino acids.

Quantitative results for four *N*-nitrosamines, each representing one of the four classes, are shown in Fig. 1. The results showed that it was possible to determine such compounds at nanogram levels with less than 5% standard deviation. The standard deviation is based on independent triplicate runs at each level. Though not shown in Fig. 1 such working curves were linear over two to three orders of magnitude.

Figure 2 shows a chromatopolarogram for class II compounds. The response is sharp and the separation excellent. Chromatopolarograms for class I and class IV compounds were reported previously (Vohra & Harrington, 1980).

The most important class III-type compound is NDELA, a nitrosamine reported to be a potential contaminant in cosmetic or other products based, in part, on triethanolamine or diethanolamine (Fan *et al.* 1977). Using the procedure described above, five com-

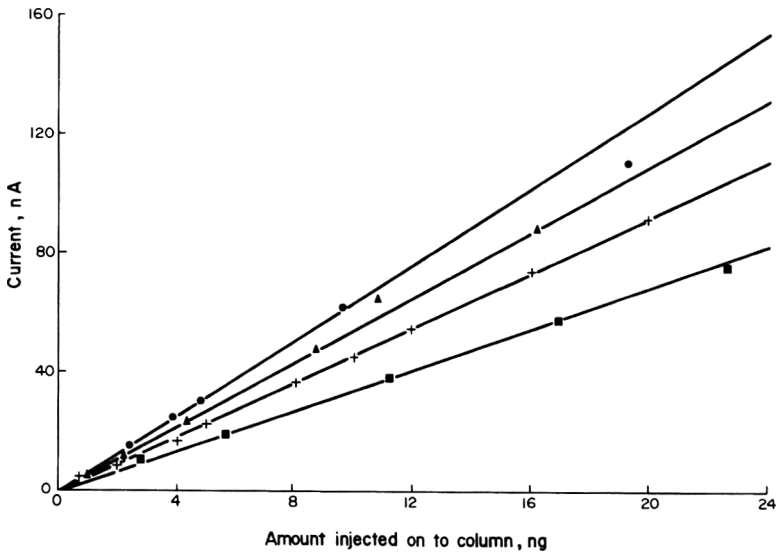

Fig. 1. Chromatopolarography of four *N*-nitrosamines. Plots of amount injected onto column against peak current for *N*-nitrosodipropylamine (+), *N*-nitroso-*N*-methylaniline (●), *N*-nitrosodiethanolamine (▲) and *N*-nitrosoproline (■). For details of chromatopolarographic conditions, see text.

Table 1. *Determination of NDELA in cosmetic products by high-performance liquid chromatography and differential pulse polarography*

Prepared concn of NDELA in sample (ppm)	Recovery of NDELA* (%) in sample no.					Corrected recovery of NDELA† (%) in sample no.				
	1	2	3	4	5	1	2	3	4	5
0·5	87	89	41	70	83	94	95	44	76	89
1·0	88	89	40	74	85	94	95	44	80	90
1·5	91	88	44	80	89	96	94	48	85	94
2·0	90	90	42	82	90	95	94	44	87	95

NDELA = *N*-Nitrosodiethanolamine

*Mean of duplicate analyses.

†Based on analyses of NDELA-containing aqueous solutions for each determination in place of the cosmetic product.

mercial cosmetic products of different formulations were analysed before and after the addition of NDELA. These products included a shampoo, various hand and face creams and a hand lotion. The results for the analyses are shown in Table 1. NDELA was added to samples of the products at concentrations ranging from 0·5 to 2·0 ppm. These levels were chosen for convenience and because they covered the permitted ranges set by regulation for total nitrosamine levels in some products, such as pesticides. Acceptable levels of NDELA in cosmetic products, have not yet been set by regulatory agencies. Figure 1 suggests that the polarographic detector is sensitive to less than nanogram amounts of NDELA. Hence, lower levels could be determined by changing the size of the sample and making appropriate changes in reagent quantities. Within the limits of the procedure used, no NDELA was detected in the pure samples of the products tested. When NDELA was added the recovery efficiencies ranged from 44 to 96%. This variation can be attributed to the wide range of ingredients used in the formulation of such products, as observed earlier by Fan *et al.* (1977). Sample no. 3 was a shampoo which consistently yielded low recoveries. This procedure for the determination of NDELA is simple and relatively rapid; work-up and analysis for a single sample requires less than 1 hr.

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ANALYSIS OF VOLATILE *N*-NITROSAMINES IN COMMERCIAL DRUGS

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Abstract—Various formulations of drugs containing aminopyramine, disulfiram or oxytetracycline were analysed for volatile *N*-nitrosamines by gas chromatography using a chemiluminescence detector. Thirty-seven samples of aminopyrine-based drug formulations contained *N*-nitrosodimethylamine at levels ranging from 1 to 900 μg/kg, and *N*-nitrosodimethylamine was found at levels ranging from non-detectable to 7·0 μg/kg in nine samples of oxytetracycline-containing drugs. Four disulfiram formulations contained *N*-nitrosodiethylamine at levels in the range 94–980 μg/kg.

Introduction

The carcinogenicity of a high proportion of *N*-nitroso compounds, now well established, has been reviewed (Magee, Montesano & Preussmann, 1976). Since the discovery some 20 yr ago that *N*-nitrosodimethylamine (NDMA) was carcinogenic in rats the methodology used to trace volatile *N*-nitrosamines in the environment has been considerably improved. Methods such as thin-layer chromatography (Preussmann, Neurath, Wulf-Lorentzen, Daiber & Hengy, 1964) or gas chromatography flame ionization detection (Williams, Timberlake, Tucknott & Patterson, 1971), which permitted detection at only mg/kg levels, first gave way to more selective and sensitive techniques using gas chromatography with selective nitrogen detection (Howard, Fazio & Watts, 1970) or electron capture detection of derivatives such as nitramines obtained by oxidation (Sen, 1970) or of heptafluorobutyryl derivatives of the parent amines obtained by reduction (Eisenbrand, 1970). This lowered the limit of detection to the μg/kg range. More recently, the use of the Thermal Energy Analyzer (TEA), proposed by Fine & Rufeh in 1974, gave a further improvement by reducing the problems of background interference. A manual of methods has been prepared by the IARC (Preussmann, Castegnaro, Walker & Wasserman, 1978) which describes in detail a number of methods of analysing for volatile *N*-nitrosamines. In 1972, a report from Lijinsky, Conrad & Van de Bogart on the possibility of nitrosation of commonly used drugs such as oxytetracycline, aminopyrine, disulfiram, niketamide and tolazamide created some concern and prompted researchers to consider the possibility of nitrosation of such drugs *in vivo* (Sander & Walz, 1976; Scheunig & Ziebarth, 1976). Subsequently evidence of the presence of NDMA was shown in aminopyrine-containing drugs (Eisenbrand, Spiegelhalder, Janowski, Kann & Preussmann, 1978) and has been confirmed by work in our laboratory. This paper reports our experience with the use of the TEA in the field of analysis of volatile *N*-nitrosamines in drugs.

Experimental

All reagents and solvents used were of analytical reagent grade. Dichloromethane was redistilled from sodium carbonate in 2-litre batches, the first 100 ml of distillate being rejected. In order to ensure the absence of contamination, a blank test was carried out using all reagents and following the analytical procedure.

Analysis was performed using a GC–TEA system. The GC used was a Pye 104 fitted with a column (6 m × 0·125 in.) packed with 15% FFAP on Chromosorb W, 80–100 mesh. The GC conditions were as follows: column temperature, 180°C isothermal; carrier gas, argon, 30 ml/min; detector, TEA from Thermo Electron Corporation, Waltham, MA, USA; temperature of the catalytic oven, 450°C.

To isolate the *N*-nitrosamine from the drugs, steam distillation of the sample diluted in water was first attempted but the samples foamed and recoveries of the internal standard, which was added to the sample before dilution, were generally very poor. Attempts to apply the method using distillation from mineral oil, already in use in our laboratory, with the modifications proposed by Eisenbrand *et al.* (1978), were more successful and recoveries were in the range of 70–90%. The frozen distillate was thawed, extracted with dichloromethane and the extract was concentrated using a Kuderna-Danish evaporator. Since direct injection of this extract caused the GC column to deteriorate a clean-up of the extract was necessary. This problem was overcome by using the two-bed alumina column described by Walker & Castegnaro (1976). The final method used was as follows. A 1-g aliquot of the sample (powdered in the case of granules or pellets) was mixed with 1 g ascorbic acid, 20 ml paraffin oil, 6 g cellulose, 10 ml distilled water,

Table 1. *Results of the analysis of NDMA in aminopyrine-containing drugs*

Type of formulation	No. of samples	Range of NDMA levels detected (μg/kg)
Syrup	6	4–300
Drops	8	1–25
Suppositories	4	200–380
Tablets	19	30–900*

NDMA = *N*-Nitrosodimethylamine
*Two additional samples were found to contain extreme levels of 36 and 90 mg NDMA/kg.

Table 2. *Results of the analysis of NDMA in oxytetracycline-containing drugs*

Type of formulation	No. of samples	Range of NDMA levels detected (μg/kg)
Tablets	4	2·1–7·0
Powder	1	2·8
Drops	1	4·2
Injection	1	5·1
Ointment	2	ND–2·8

NDMA = *N*-Nitrosodimethylamine ND = Not detected

0·5 ml 5 N-sulphuric acid and the internal standard. The mixture was distilled under vacuum and the distillate was trapped in a glass flask immersed in liquid nitrogen. The distillate was allowed to thaw and 2 ml 0·2 N-NaOH were added. The distillate was extracted three times with 10 ml dichloromethane. The extract was dried with about 30 g sodium sulphate, taken up in 2 ml hexane and subjected to adsorption chromatography on a two-bed alumina column made up of 3 g basic alumina activity 2 and 3 g neutral alumina activity 3. The column was eluted with the following sequence of eluants: 25 ml 25% (v/v) diethylether in pentane; 15 ml 50% (v/v) diethylether in pentane; 15 ml 25% (v/v) diethylether in pentane; 15 ml diethylether. The eluate was concentrated to about 10 ml using a Kuderna-Danish evaporator and aliquots were used for GC–TEA.

Results

A list of drug formulations that might possibly contain volatile *N*-nitrosamines was compiled from the *Vidal* (the French dictionary of drugs acceptable for current use). The drugs examined included aminopyrine and oxytetracycline, from which NDMA can be produced by nitrosation, and disulfiram, from which *N*-nitrosodiethylamine (NDEA) can be formed (Lijinsky *et al.* 1972). Samples were purchased from various pharmacies in Lyon and the results of analysis of aminopyramine- and oxytetracycline-containing drugs in which NDMA was found are shown in Tables 1 and 2. All four disulfiram formulations tested were shown to contain NDEA in the range 94–980 μg/kg.

Discussion

When analysing for *N*-nitrosamines in drug formulations such as drops, syrups, injections and suppositories no problems of reproducibility of the results were encountered, but variations were found when analysing tablets of aminopyrine. This is not due to the method of analysis but to sampling problems, since the levels of *N*-nitrosamines were found to vary very considerably between individual tablets. However good reproducibility could be obtained by grinding and careful homogenization using a complete box of tablets and by sampling aliquots. The results reported represent values obtained by this method of sampling.

A potential weekly intake by an individual of volatile *N*-nitrosamines from these drugs has been calculated on the basis of the prescribed daily dose and the data obtained in this study. Thus intake of NDMA with oxytetracycline-containing drugs could be in the order of 0·4–2 μg NDMA/wk and from disulfiram 0·5–5 μg NDEA/wk. If the drugs are taken for long periods these levels, particularly that for NDMA, could be considerably more than the 1 μg/wk calculated by Gough, Webb & Coleman (1978) to be the average volatile dialkyl nitrosamine intake from food and they would be comparable with that resulting from the consumption of alcohol in North-West France as calculated by Walker, Castegnaro, Garren, Toussaint & Kowalski (1979) from epidemiological nutritional data. Whether such levels could be of significance in human cancer would be difficult to determine from an epidemiological study since the *N*-nitrosamine content of the drugs as purchased from a pharmacy is rather variable. Furthermore the drug disulfiram will only be taken by the relatively small population of drinkers under treatment for alcoholism. Nevertheless in evaluating the effect of the intake of *N*-nitrosamines in humans it would seem important that epidemiological inquiries should include an attempt to assess the intake of nitrosatable drugs.

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REDUCTIVE DESTRUCTION OF *N*-NITROSODIMETHYLAMINE AS AN APPROACH TO HAZARD CONTROL IN THE CARCINOGENESIS LABORATORY

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Abstract—One-step conversion of both *N*-nitrosodimethylamine and 1,1-dimethylhydrazine to dimethylamine was effected rapidly and quantitatively in various solvents using nickel-aluminium alloy in the presence of aqueous alkali.

Introduction

N-Nitrosodimethylamine (NDMA) is widely used in biological research (Magee, Montesano & Preussmann, 1976) as well as chemical studies (Seebach & Enders, 1975). Because NDMA is carcinogenic (IARC Working Group, 1978), a reliable means of destroying it should be available for a wide variety of hazard-control applications associated with its use, including rendering it innocuous in waste materials prior to disposal.

Gangolli, Shilling & Lloyd (1974) have recommended that basified aqueous solutions of NDMA and related nitrosamines be degraded by treatment with aluminium foil. However, we have found (Emmett, Michejda, Sansone & Keefer, 1980) that, while the NDMA is indeed quantitatively consumed in this system, the primary product of the reaction is 1,1-dimethylhydrazine, which is itself a carcinogen in mice (IARC Working Group, 1974). Recently, Seebach & Wykypiel (1979) have recommended a useful two-step procedure for completely reducing nitrosamines to the less toxic amines first by converting the nitrosamine to the hydrazine with lithium aluminium hydride in an anhydrous ethereal solvent and then by hydrogenolysing the hydrazine to the amine with Raney nickel under hydrogen.

Taken together, the data of Gangolli *et al.* (1974), Seebach & Wykypiel (1979) and Emmett *et al.* (1980) led us to postulate that treatment of nickel–aluminium alloy with hydroxide ion, the standard procedure for preparing Raney nickel (Augustine, 1965), should result in nitrosamines present in the solution being reduced completely to amines in one step. Similar one-step procedures have been used to reduce other organic functional groups (see, for example, Schwenk, Papa, Hankin & Ginsberg, 1955). This paper summarizes the data obtained in testing our hypothesis. Our results indicate that the nickel–aluminium–alkali system is a substantial improvement over procedures previously recommended for destroying NDMA in solution.

Experimental

In a typical experiment, 10 μl NDMA was dissolved in 2 ml 0·5 M-potassium hydroxide solution and treated with 100 mg nickel–aluminium alloy (Alfa Divn, Ventron Corp., Danvers, MA).

Aliquots of 0·5 μl were analysed at various times during the course of the reaction by direct injection into a Hewlett Packard 5830A Gas Chromatograph equipped with a 2 mm (ID) glass column, 1·83 m long, packed with 10% Carbowax 20 M plus 2% potassium hydroxide on Chromosorb W-AW. Column temperature was maintained at 50°C, and the nitrogen flow rate was constant at 19 ml/min. The flame ionization detector peaks were automatically quantitated by the instrument's integrator unit, and the integrals for each peak were compared with those of independently prepared standard solutions. Retention times for the standard peaks were 0·6 min for dimethylamine, 1·4 min for 1,1-dimethylhydrazine and 17 min for NDMA under these conditions.

Several variants of the above reaction were conducted. In one case, independently prepared 1,1-dimethylhydrazine was used in place of the NDMA. In other cases, 11 mg aluminium foil was used in place of the alloy. In still other experiments, the aqueous solvent was replaced by methanol–water (9:1, v/v), by dichloromethane mixed with an equal volume of M-potassium hydroxide and three volumes of methanol to give a homogeneous solution, or by 0·5 ml mineral oil diluted with 0·5 ml hexane and then mixed with 1 ml M-potassium hydroxide solution. In the latter case, two distinct layers were maintained and both phases were sampled for gas-chromatographic analysis.

When reduction was complete, the resulting slurry was suction-filtered through Celite, care being taken not to let the potentially pyrophoric filter cake dry in the presence of organic solvents or other flammable materials. We preferred to recycle the spent nickel, or else to discard it carefully with the solid waste after autoxidation was demonstrably complete (Enders,

Pieter, Renger & Seebach, 1978), but it may also be dissolved cautiously in mineral acid for disposal (Schwenk *et al.* 1955).

Results

Destruction of NDMA was very rapid ($t_{\frac{1}{2}}$ *c.* 2 min) in the typical reaction mixture. 1,1-Dimethylhydrazine was observed as an intermediate but was destroyed as the reduction progressed. The final reaction mixture, analysed after 1–2 hr, showed only dimethylamine, with no trace of 1,1-dimethylhydrazine or NDMA. Independently prepared 1,1-dimethylhydrazine was, in a separate experiment, converted rapidly ($t_{\frac{1}{2}}$ *c.* 4 min) and quantitatively to dimethylamine under these conditions.

When the alloy was replaced by aluminium alone, dimethylamine was the minor product (13%) and 1,1-dimethylhydrazine the major product (83%). In contrast to our earlier experience (Emmett *et al.* 1980) using a different gas-chromatographic column, significant peaks were not found at the retention time corresponding to 1,2-dimethylhydrazine, suggesting that some other constituent(s) of the final reaction mixture might have been responsible for the small peak observed in the earlier work at a retention time of 4 min.

When nickel–aluminium alloy was added to solutions of NDMA in methanol–M-potassium hydroxide (9:1, v/v), the nitrosamine was again quantitatively reduced to dimethylamine, but reaction was slower ($t_{\frac{1}{2}}$ *c.* 60 min). In dichloromethane–M-potassium hydroxide–methanol (1:1:3, by vol.), destruction was again rather slow ($t_{\frac{1}{2}}$ *c.* 35 min) but complete. In mineral oil–hexane–M-potassium hydroxide (1:1:2, by vol.), reduction in the two-phase system appeared to be as rapid as in the original aqueous medium ($t_{\frac{1}{2}}$ *c.* 2 min).

Discussion

A research laboratory in which nitrosamines are being manipulated typically generates potentially carcinogenic refuse in a wide variety of forms, including unused drinking-water solutions from animal bioassay experiments, rinsings from distillation apparatus and aged analytical standards. A reliable means of destroying all carcinogenic components of this refuse before its release into the general environment must be available. Complete oxidative degradation in an efficient incinerator would seem to be the most general approach to destruction of organic carcinogens, but this method can be impractical, especially for large volumes of non-combustible (e.g. aqueous) materials.

Our success in converting NDMA quantitatively to relatively non-toxic dimethylamine in a variety of solvents commonly used with nitrosamines (including water, mineral oil and dichloromethane) suggests that the nickel–aluminium–alkali system may be the basis for a broadly applicable approach to hazard control for work with nitrosamines in the carcinogenesis research laboratory. Future work will explore the effects of variations in structure and other parameters on the course of this reaction, with the ultimate goal of developing detailed procedures.

Note—All reactions were conducted in 2-ml reaction vials at room temperature in a hood. These reactions were performed as feasibility studies only and should not be read as detailed products. Great care should be exercised with scaled-up reactions.

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Review Section

REVIEWS OF RECENT PUBLICATIONS

Metal Contamination of Food. By C. Reilly. Applied Science Publishers Ltd, London, 1980. pp. xvi + 235. £17.00.

The part metals play in human nutrition and health is becoming increasingly recognized. Only a few metals can justifiably be called nutrients, the vast majority of those occurring in food being either biologically indifferent or potentially toxic. Concerned with the less desirable metals, this compact volume is written in two sections; the first is general in nature, the second more specific.

In Part I a broad examination of metals in the environment, food and the human body is followed by a description of the various ways by which food contamination can occur. The metal content of animal fodder crops, largely determined by the characteristics of the ambient soil and subsoil, can be influenced by the ability of certain plants to take up an excess of specific elements. For example, the accumulation of selenium by the legume *Astragalus racemosus* has been reported to result in levels approaching 15 g/kg of plant tissue. However, it is unlikely that these inflated amounts will be transferred to man unless the plant material is used directly for human consumption. This was the case when rice that was contaminated through irrigation of the crop with water industrially polluted with cadmium caused cadmium-induced disease in hundreds of people and over 50 deaths in Toyama City, Japan, in the 1950s.

Other factors affecting the metal composition of crops, including wind and waterborne pollutants and the use of municipal sewage, fertilizers and other chemicals on agricultural land are discussed, along with the opportunities for contamination that arise during the production, harvesting, cooking, processing and storage of foods. Completed by a chapter on analytical methodology and instrumentation, Part I also presents a consideration of various relevant aspects of food quality control and legislation (in the UK and overseas).

In Part II, comprehensive summaries of data from many sources give information on the chemical and physical properties, production and uses, biological activity, environmental, food and body levels and analytical methods for 22 metals that are generally considered undesirable. A final section briefly outlines similar data on the remaining metals at variable levels in foods.

Written in a clear, readable style, with an extensive reference list for each chapter, this book will be of value to those concerned with the biological sciences and the food industry. It may also prove of interest to the layman, aware of the dangers of heavy metals and concerned about the part metals in food may play in people's lives.

Toxic Constituents of Plant Foodstuffs. 2nd Ed. Edited by I. E. Liener. Academic Press, Inc. (London) Ltd, London, 1980. pp. xiv + 502. £27.80.

The introduction to this, the second edition of the very useful original *Toxic Constituents of Plant Foodstuffs*, is followed by a collection of twelve up-dated monographs, each written in precise terms and illustrated with clear diagrams and tables. The book is usefully completed with an extensive reference list and full index.

In the early part of the book the characteristics, occurrence, mechanisms of action and toxicity of protease inhibitors, haemagglutinins (lectins), glucosinolates, cyanogens, saponins and gossypol are considered, group by group. The relatively short chapter on cyanogens, for example, includes a discussion of human metabolism of inorganic cyanide in addition to the section on the toxicology of cyanogenetic plants. The dietary sources of cyanogens, their chemistry and the methods of extracting and analysing them are also described.

Subsequently four types of toxic constituents are discussed according to their effects. The section on lathyrogens includes information on the chemistry, toxicity and mode of action of the osteo- and neurolathyrogens and their biosynthesis, analytical detection and estimation. The use of *Lathyrus sativus* as a food is examined with particular reference to the social and economic aspects, the amino-acid composition of the bean and the methods for removing the toxin from the bean. The epidemiologic, genetic and enzymologic aspects of favism, and its mechanism of action are considered in the next chapter which is followed by a description of the symptoms and factors influencing the clinical disorders induced by allergens in the diet, including their characteristics, immunological properties, and detection. The fourth chapter of this group deals with the carcinogens associated with plants, such as mycotoxins and other microbial products, cycasin, the pyrrolizidine alkaloids and others.

The toxic factors induced by processes such as treatment with heat, acid, alkali, bleaching agents, irradiation, solvents and fumigants are the subject of a further monograph. The remainder of the topics covered are brought together under the title "Miscellaneous Toxic Factors" and include oestrogenic factors, stimulants, depressants, hypoglycaemic agents, toxic amino acids, antivitamin factors, anti-enzymes, metal-binding constituents, tannins, flatus-producing factors and a selection of other plants with toxic constituents.

This comprehensive volume should prove to be of value and interest to a wide audience of readers both as a reference text and as an introduction to the current literature at the start of an in-depth study of a specific plant toxicant.

Environmental Health Criteria. 15. Tin and Organotin Compounds: A Preliminary Review. Published under the joint sponsorship of the United Nations Environment Programme and the World Health Organization. WHO, Geneva, 1980. pp. 109. Sw.fr. 7.00 (available in the UK through HMSO).

This review of tin and organotin compounds is a very comprehensive and up-to-date one. There are useful sections on the chemistry, analysis, environmental concentrations and exposures, and metabolism of both inorganic and organic tin compounds. Of particular interest are the sections dealing with the organotin compounds. Animal experiments relating to both local and systemic effects of the organotins are summarized and the differing effects of mono-, di-, tri- and tetrasubstituted organotin compounds are discussed. In general, mono- and diorganotins, used mainly in the plastics industry, are less toxic than triorganotin compounds which are used in fungicides, insecticides, and other biocidal chemicals. Tetrasubstituted organotin compounds are used mainly as intermediates in the production of other organotin compounds, and tend to resemble the tri-substituted compounds toxicologically. Although experiments have demonstrated that both dioctyl and dibutyl tin can cause thymic atrophy in rats and that some trialkyltin compounds produce a characteristic lesion in the central nervous sytem of experimental animals, little is known about the mechanism of action of these compounds and it is recommended that more information should be obtained. Information regarding bioconcentration is also needed. Further investigation of the possible methylation of tin by organisms present in the environment would be of particular interest.

Compared with most organotin derivatives, inorganic tin and its salts are not highly toxic, mainly because of their poor absorption and rapid tissue turnover. However, soluble tin salts are gastric irritants and high doses of inorganic tin compounds seem to affect the central nervous system.

As well as the studies on experimental animals, the effects of inorganic and organic tin compounds on man are discussed, although there are comparatively few clinical observations concerning tin compounds. Episodes of acute poisoning by inorganic tin have been reported, mainly in association with the ingestion of fruit juices containing high concentrations of tin. Exposure by inhalation of tin(IV) oxide is also known to cause stannosis, a benign condition of pneumoconiosis. Toxic lesions of the skin among laboratory and process workers handling di- and tributyltin compounds have been reported together with cases of accidental organotin poisoning.

Environmental Carcinogens. Selected Methods of Analysis. Vol. 3. **Analysis of Polycyclic Aromatic Hydrocarbons in Environmental Samples.** Edited by M. Castegnaro, P. Bogovski, H. Kunte & E. A. Walker. IARC Publications No. 29. International Agency for Research on Cancer, Lyon, 1979. pp. ix + 240. Sw.fr. 50.00 (available in UK from HMSO).

Polycyclic aromatic hydrocarbons represent an important group of chemical carcinogens in the human environment, and wide ranging research has been directed towards investigating their formation and presence in the environment, their role in experimental oncology and their epidemiological association with cancers in man.

This volume, the third in a series on analytical methods for environmental carcinogens, consists of three main sections together with an introductory review chapter on the carcinogenicity of polycyclic aromatic hydrocarbons. The first section, which consists of three chapters, starts with the important and at times perplexing topic of the nomenclature and structure of polycyclic aromatic hydrocarbons. The second and third chapters deal with the sources and occurrences of polycyclic aromatic hydrocarbons and with the distribution of these compounds in environmental specimens. Section 2, comprising five chapters, deals with procedures for the collection of specimens, with techniques for isolating polycyclic aromatic hydrocarbons from other substances likely to interfere with their analysis, and with methods of analysis and of avoiding the introduction of contaminants in analytical solvents.

In the final section detailed descriptions are given of eight analytical methods using chromatographic and spectroluminescence techniques for the detection and determination of polycyclic aromatic hydrocarbons in a variety of materials ranging from water and automobile exhaust gases to various foodstuffs.

This volume maintains the high standards of presentation and attention to detail set in the earlier volumes (*Cited in F.C.T.* 1979, **17,** 535; *ibid* 1980, **18,** 307) and should be of considerable value to analytical chemists involved in the determination of this important class of environmental carcinogens.

Hazards to your Health. The Problem of Environmental Disease. By J. Gorman. New York Academy of Sciences, New York, 1979, pp. vii + 70. $4.00.

Persistent Poisons. Chemical Pollutants in the Environment. By M.-J. Schneider. New York Academy of Sciences, New York, 1979. pp. vi + 67. $4.00.

The Asbestos Hazard. By P. Brodeur. New York Academy of Sciences, New York, 1980. pp. vi + 93. $4.00.

Ignorance is bliss. One can only assume that the reluctance of both industry and government in this country to educate the general population about the possible toxicological hazards of life arises out of their kindness. In the USA, land of open government, things are a little different. The man in the US street is not subjected to the same paternalistic protection, and has greater opportunities both to learn about and to contribute to the often heated discussions about the threats (both real and imagined) to his health.

In June 1978, the New York Academy of Sciences organized a Science Week entitled "The Scientific Basis for the Public Control of Environmental Health Hazards". The crop of publications named above aims to present for the layman the essential issues

discussed at three conferences that took place during that week. *Hazards to your Health* focuses on the present scientific and political difficulties associated with the assessment of carcinogenic risk. The halogenated aromatic hydrocarbons, featuring such unwelcome environmental guests as dioxin, DDT, polychlorinated biphenyls and polybrominated biphenyls are covered by *Persistent Poisons*, while the current nightmare of the industrial sphere comes to the fore in the third of the trilogy, *The Asbestos Hazard*. The three authors argue a case for greater governmental involvement and, by and large, this case is presented convincingly, and the typical consumerist mistake of over-interpreting available data is avoided. The principal concern is to convince the lay audience of the wide gaps in our present knowledge; caution is the message. Yes—recommended reading for the thinking layman.

[For those of more scientific bent the full proceedings of each of these three international conferences were published as volumes 329, 320 and 330 respectively, of the *Annals of the New York Academy of Sciences*.]

Toxicology and Occupational Medicine. Edited by W. B. Deichman. Elsevier/North-Holland Inc., Amsterdam, 1979. pp. xiv + 480. $55.00.

This book, the fourth volume in a series on Developments in Toxicology and Environmental Science, constitutes the proceedings of the Tenth Inter-American Conference on Toxicology and Occupational Medicine held in Florida in October 1978.

The subject matter is extremely diverse, ranging from several papers on experimental design and methodology in toxicology to specific studies in human populations relating to occupational exposures. Included on the way are some detailed studies of biochemical and pathogenic mechanisms of specific chemicals, including styrene, pentachlorophenol and dioxin, and such exotica as the ciguatera syndrome and the study of lumbar musculature as a guide to weight-lifting ability.

The book, which appears to have been produced as economically as possible but still costs $55, is packed with information of considerable relevance to current toxicological issues. Papers on "Mechanism of action of diet as a carcinogen", "Structure–activity relationships amongst the primary aromatic amines in the induction of bladder cancer" and "The metabolism/pharmacokinetics of pentachlorophenol in man, and a comparison with the rat and monkey", amongst many others, illustrate the broad interests covered. The book is, of course, particularly strong at the interface of toxicology and occupational medicine, a topic explored in some depth by a number of contributors. However, environmental studies are not ignored.

This book is strongly recommended to all toxicologists who wish to make contact with the whole range of their subject, and to those in industrial medicine who have an interest in the rich contributions toxicology can make to the understanding of their practice.

Reactions of the Skin to Cosmetic and Toiletry Products. Consumers' Association, London, 1979. pp. v + 155. £50.00 (from Consumers' Association, Caxton Hill, Hertford).

This report presents the results of a study aimed at assessing the true incidence of adverse reactions of skin to cosmetics and toiletry products in the adult population of the UK, with a view to determining whether full ingredient listing would benefit consumers. It was the first large-scale study of its kind in this country and the results seem to confirm what many must have long suspected, in terms of the incidence of irritant or allergic reactions in relation to age, sex, the type of products involved and the severity of the reactions, although that in itself does not detract from the significance of the study. The conclusion reached regarding the issue of full ingredient labelling was that this would not really help members of the public who suffer from irritant or allergic reactions to cosmetics since the precise nature of the offending ingredient(s) often remains unidentified. It was argued that even in cases of established allergy to one of the more common sensitizers, adequate protection could be afforded by implementation of revised EEC requirements on labelling. The Consumer Association stance, nevertheless, is to continue in favour of full ingredient listing on these products on the grounds that the consumer has a right to know what he or she is purchasing. The difficulties this requirement may present to the manufacturers of cosmetics are generally given fair consideration, except that the problem of keeping up with minor formulation changes necessitated by shortages of raw materials is not considered at all.

The survey itself was carried out in three stages. Initially 11,062 people were interviewed during a period of 5 months in late 1975 and early 1976. Of these, 12% claimed to have had an adverse response to a cosmetic in the previous 12 months. This group was then asked to complete a more detailed postal questionnaire. The 626 replies were analysed in depth for information on the products involved, the nature, severity and duration of the reaction and the action taken. In the third stage, 1022 individuals in one parliamentary constituency were interviewed; 85 of these claimed to have had an adverse reaction in the preceding 12 months. Patch testing was then carried out on 44 of the individuals claiming reactions, in an attempt to distinguish between irritant and allergic contact reactions and to assess the degree of 'overclaiming'. Unfortunately the researchers were unable to achieve these last aims on what was admitted to be a very small sample.

It was shown that 34 of the 44 people patch-tested had a genuine reaction to cosmetics, and of these, 11 were found to be allergic reactions. Extrapolating from these data it was suggested that between 3 and 9% of the adult population in this country may suffer an adverse reaction to a cosmetic in any year, that the majority of these reactions are likely to be of an irritant type, but that 1–3% of the population may suffer an allergic contact dermatitis. Perfume was thought to be responsible in at least three cases of sensitivity, involving a deodorant, a lipstick and a proprietary

perfume product. Lipsticks were involved in two other cases, in both of which the allergen was thought to be eosin, no longer in general use in lipsticks. Phenylenediamines were implicated in one case of allergy to a hair dye, but the allergens in the remaining five of the 11 cases of confirmed sensitivity to a product (four skin creams, one deodorant) could not be identified from the battery of 24–36 common sensitizers tested. In each of the three surveys, the products most frequently mentioned as causing an adverse reaction were soaps, deodorants, skin creams and eye make-up. Patch tests revealed that these products, together with after-shaves and shampoos, were largely responsible for irritant reactions but that perfumes may often be at the root of allergic problems.

A minor criticism of the presentation of this report concerns the tabulation in Appendix VI of data from the first two stages of the survey. There are 18 tables, many covering at least four pages and one extending over ten pages. It would have helped the reader if the page numbers in the rest of the book had been continued over the 72 pages of this appendix and if the tables themselves had been included on the contents page.

Progress in Drug Metabolism. Vol. 3. Edited by J. W. Bridges & L. F. Chasseaud. John Wiley & Sons Ltd, Chichester, 1979. pp. ix + 372. £19.25.

This book is the third volume of a series which aims by critical reviews to keep the reader up to date in developments in various aspects of xenobiotic metabolism. The present volume consists of six reviews each with a list of references together with a subject index for the entire volume.

Three of the chapters are devoted to techniques, namely high-pressure liquid chromatography (D. A. Schooley and G. B. Quistad), analysis of drugs in biological fluids (W. Riess, S. Brechbuhler and J. P. Dubois) and applications of nuclear magnetic resonances spectroscopy in drug metabolism (I. C. Calder). The first of these deals with the theory of high-pressure (performance, or even price!) liquid chromatography, instrumentation, columns, and sample derivatization and then follows on with a series of examples of the analysis of various pesticides. The chapter on drug analysis in biological fluids reviews the various techniques available including thin-layer, gas and high-pressure liquid chromatography together with gas chromatography–mass spectrometry, double radioisotope derivative techniques and radioimmunoassay. A further section is devoted to attempts that have been made to correlate plasma levels of tricyclic antidepressant drugs with observed therapeutic effects. In the chapter on the use of nuclear magnetic resonance spectroscopy in drug metabolism the basic principles underlying this technique are described together with examples of its use in identifying the products of both xenobiotic biotransformation and conjugation reactions.

The chapter by F. Oesch is devoted to the important enzyme epoxide hydratase (epoxide hydrase, EC 3.3.2.3) which converts potentially toxic epoxides into dihydrodiols. The species and tissue distribution, induction, purification and properties of this enzyme are considered together with its biological role with special reference to effects on the mutagenicity of xenobiotics. The chapter by G. T. Brooks deals with the metabolism of xenobiotics in insects and includes a discussion of the properties and functions of insect cytochrome *P*-450. Attention is also devoted to the design of selective insecticides. Following on from insect metabolism the chapter by D. H. Hutson deals with the metabolism of synthetic pyrethroid insecticides in mammals. Although a large number of compounds are covered, only brief attention is given to species differences in metabolism and to the relationship between metabolism and toxicity.

As with the previous volumes of this series (*Cited in F.C.T.* 1977, **15**, 239; *ibid* 1978, **16**, 283) this book provides reviews of a high standard. The subject index is useful and the text is well illustrated with line drawings indicating chemical structures and metabolic pathways. In such a series the usefulness of any particular volume to a reader is obviously dependent on the subjects reviewed. Since half of the chapters in this volume deal with pesticides and their metabolism and analysis, the book should be of particular appeal to those working in this area of xenobiotic metabolism. It is unfortunate that whilst this series is to be recommended, the price of the volumes will probably mean that it is not purchased for personal use.

Industrial and Environmental Xenobiotics. In vitro Versus in vivo Biotransformation and Toxicity. Proceedings of an International Conference held in Prague, Czechoslovakia, 13–15 September 1977. Edited by J. R. Fouts & I. Gut. Excerpta Medica, Amsterdam, 1978. pp. xvii + 345. Dfl. 130.00.

These proceedings of an international conference held in Prague in 1977 illustrate the number of techniques available for the *in vitro* evaluation of xenobiotic metabolism and the host of problems associated with the subsequent extrapolation of the data to the whole animal. Concerned principally with the fast-moving field of metabolism, the contributions successfully, if briefly, consider the clinical, occupational and environmental toxicity of a wide variety of chemicals and drugs. Many authors are concerned with ascertaining the predictive value of studies in cultures of isolated cells or organ tissues, and focus in particular upon the difficulties involved in relating the results obtained to the situation *in vivo*.

An excellent short review by I. Gut highlights the limitations intrinsic to the application of a theoretical analysis of xenobiotic metabolism to the factors affecting the metabolism *in vivo*. J. R. Withey provides convincing evidence for the role of pharmacokinetic analysis in toxicology. All too often in the past, failure to consider this in sufficient detail has resulted in poor extrapolation from *in vitro* to *in vivo* situations and an inability to make an accurate estimation of the toxicological effect. This author emphasizes the need for a single-dose pharmacokinetic study before and at least once during a chronic toxicity study, with the aim of establishing and monitoring dose regimens that both achieve and maintain a steady state throughout the test. Pharmacokinetic analysis is considered frequently in these proceedings. For example,

H. de Nijs and C. J. Timmer use a comparison of data obtained from liver perfusion studies with those derived from the anaesthetized rat to make a quantitative prediction of the liver's contribution to total body clearance of a drug.

It is always of interest to see the emergence of new work on the established hepatotoxins, carbon tetrachloride and ethanol. A. Tigyi and his colleagues consider the effects of carbon tetrachloride on liver nuclear-protein kinases and RNA polymerases, whilst C. S. Lieber's group discusses the microsomal and mitochondrial responses associated with the accelerated ethanol metabolism and enhanced acetaldehyde production that results from chronic ethanol consumption by rats.

The range of this volume is illustrated by the diverse section headings: (I) Chemical and biochemical models, (II) *In vitro* and *in vivo* systems in the study of xenobiotic metabolism, (III) Occupational and environmental aspects of xenobiotic metabolism and (IV) Mechanisms of toxic action. In all it is a stimulating book—not light reading, although perhaps at times a little too brief, but none-the-less an excellent conference record and a welcome attempt to focus multidisciplinary attention on the ultimate toxicological problem, namely the successful extrapolation of *in vitro* data to man and animals.

I just wish the print size and type didn't vary so much!

Selected Methods in Cellular Immunology. Edited by B. B. Mishell & S. M. Shiigi. W. H. Freeman & Co., San Francisco, 1980. pp. xxix + 486. £17.70.

For the experimental immunologist this is a most useful handbook. It includes lucid and comprehensive descriptions of all the *in vitro* laboratory techniques currently in use, and in addition discusses new areas such as immunoglobulin-producing hybrid cell lines and solid-phase radioimmune assays.

The first section discusses the various assay systems now used to evaluate *in vitro* immune responses. The introductory chapter describes the preparation of cell suspensions such as those of spleen, lymph node and thymocyte cells, the determination of cell viability and the execution of other procedures that must be carried out before the assays can be undertaken. Systems for studying the generation and regulation of immune responses *in vitro* are discussed in another chapter. Such culture systems are particularly useful for comparing the effects of immunoregulatory substances, because both the control and experimental cells are derived from one cell suspension. These methods are especially well suited to the study of how macrophages function. Other systems described include the haemolytic plaque assays which measure the number of cells secreting IgM and IgG antibodies, and the extension of these techniques to the detection of cells secreting antibodies to polysaccharides, haptens or heterologous proteins. Elsewhere, culture methods for the generation and estimation of cytolytic T lymphocytes are described, as well as procedures for inducing and measuring proliferation in response to mitogens and antigens, particular attention being paid to the variables inherent in these methods.

Another section enumerates cell separation techniques, essential for the identification of subpopulations of cells and the analysis of their function. Various cell separation methods are described in detail, including those based on the differential adherence properties of cells, on sedimentation rates reflecting cell size and density, and on differentially expressed cell surface markers.

A well-planned section on the preparation and testing of antisera includes the methodology for producing antisera to T cell surface antigens and to immunoglobulins, as well as the purification of these sera by affinity chromatography and procedures for preparing fluorescent and other such detector-coupled antibodies. The production of monoclonal antibodies using cell-fusion techniques is also described in a lucid step-wise fashion.

This book is a fine laboratory guide; all the methods are fully described and are up-to-date. It also provides essential background and reference material and, whilst concentrating on murine cells, will be extremely helpful to all immunologists who wish to apply cellular immunological methods to their work.

Inbred Strains in Biomedical Research. By M. F. W. Festing. The Macmillan Press Ltd, London, 1979. pp. xii + 483. £25.00.

The use of inbred strains is increasing rapidly at present as biomedical research workers become more familiar with the methodology and strategy involved in their usage.

Inbred strains have often been maligned as a poor model of genetically outbred populations such as man. However as Michael Festing mentions "the modern 'albino' laboratory mouse or rat maintained in a plastic cage, fed a standard pelleted diet in controlled environmental conditions, often housed in single-sex groups and protected from disease, can hardly be called 'natural' ". These outbred animals are valuable in research because so many variables are controlled to eliminate their possible influence on experimental outcome. The fact that in addition individuals of an inbred strain are genetically identical controls another experimental variable.

Section 1 of the book contains a comprehensive review of inbreeding and its consequences and of the history of inbred strains, including the development of the alloantigen hypothesis, and research into the *H*-2 histocompatibility complex and cell-mediated immune responses. The techniques used in genetic quality control are extremely well documented, but it is a pity that the quality of the photographic plates is so poor.

The text is written with a commendable economy of words and each chapter has a brief but informative introductory section together with a full description of each photomicrograph. Some organ systems are given better treatment than others, the emphasis probably reflecting the individual preferences of the authors. However, this criticism is equally true of more standard histology texts illustrated by photomicrographs taken using light or transmission electron microscopy.

In addition to revealing the mysteries of the nomenclature used in the classification of inbred strains, the strategy and possible future trends in their usage are reviewed. Using examples, Dr Festing illustrates how the value of inbred strains depends on making the most of their valuable properties whilst avoiding the pitfalls of their misuse. He emphasizes how generalizations based on inbred material must be treated with caution, since the inbred strain represents a single genotype. However, the in-depth study of such a strain may generate hypotheses for further testing. The general applicability of a study with inbred strains can be increased by using several different strains; in contrast, in some types of investigation inbred strains are unsuitable.

The second section of the book documents the inbred strain characteristics not only of the commonly used laboratory species but also of chicks, amphibia and fish. The known characteristics of each strain are given along with a comparative index to other strains used in the investigation of the same parameter. Although much of this section is devoted to mice this is to be expected since the production of inbred strains in this species is most advanced. However, an increase in the number of inbred strains of rat over the next 10–15 years is forecast.

An appendix provides data on strain rankings for various parameters in laboratory animal studies, enabling in the first instance the selection of a strain to maximize the chance of detecting an experimentally induced effect. The parameters considered for each strain are behaviour, life-span and spontaneous disease, normal physiology and biochemistry, anatomy and morphology, response to drugs, infection, immunology and reproduction.

In all, this book is a work of considerable complexity which has been elegantly, clearly and concisely compiled to provide an invaluable reference for the selection and understanding of the use of inbred strains in biomedical research.

Tissues and Organs: A Text-atlas of Scanning Electron Microscopy. By R. G. Kessel & R. H. Kardon. W. H. Freeman & Co., Ltd, San Francisco, 1979. pp. ix + 317. £20.70 (hard cover), £8.10 (soft cover).

Of the comparatively few atlases devoted primarily to the use of the scanning electron microscope (SEM) in the study of mammalian tissues, this must undoubtedly be the best as far as its coverage and the quality of the photomicrographs is concerned. The scope of the book is as applicable to the student of functional anatomy as to the serious research worker.

No series of lectures on histology can be considered complete without a section on the use of the SEM in the interpretation of biological function. It is to be regretted that the rational use of the SEM in the teaching of histology and in particular of pathology has been limited in the past, since the structure of fairly complex biological structures, seen in two dimensions using the light and transmission electron microscope, is undoubtedly easier to comprehend in conjunction with the three dimensional image afforded by the SEM.

In the past a major stumbling block to the use of the SEM in the study of mammalian tissue has been the difficulty of adequately preparing the tissue for photography. It may be for this reason that previous texts on the SEM have restricted themselves to animal specimens requiring relatively simple preparation—we can all recall the extremely attractive scanning photomicrographs of insect eyes and antennae and so on. However, the technology of specimen preparation has progressed considerably and the authors have obviously managed to prepare a vast array of mammalian tissues to an extremely high standard. To aid the newcomer to the SEM the authors have also included a useful, if short, appendix describing a preparation technique for mammalian tissue. Their own methods for revealing specific internal constituents of various tissues are often also outlined in the description that accompanies each photomicrograph.

Any atlas of morphology is only as good as its photographs, and both the quality and the reproduction of the majority of photomicrographs in this volume will be the envy of many microscopists who have encountered the problems associated with the use of the SEM in the study of soft tissues. Some of the scanning photomicrographs are accompanied by extremely good line drawings but their use is only occasional and the inclusion of more, particularly for some of the more complex organs, would have been advantageous, especially as far as the teaching capacity of the book is concerned.

Where appropriate the authors have also included photomicrographs of tissues taken using the transmission electron microscope in order to illustrate the finer points of interpreting the accompanying scanning photomicrographs. Their inclusion is indicative of the necessity of combining the use of light, transmission electron and scanning electron microscopy rather than taking the more insular stands previously adopted by workers in these three related disciplines.

Another valuable feature of the book is the use of photomicrographs of polymer replicas of the vascular systems of organs and tissues. Although the preparation of such casts is not a new procedure, its marriage with the SEM certainly adds considerably to the visualization of the extensive and complex vascular networks associated with different organs.

The authors of this book must undoubtedly feel a good measure of pride in its outcome. One hopes that this volume on normal tissue organization will be followed by a series on the pathology of tissues as revealed by the SEM.

Fundamentals of Human Lymphoid Cell Culture. By J. L. Glick. Marcel Dekker, Inc., New York, 1980. pp. x + 157. Sw.fr. 52.00.

This book describes the establishment of human lymphoid cell lines, and the various methods used for their initiation and maintenance. A common procedure for inducing the establishment of human lymphoid cells is to expose the primary cultures to Epstein-Barr virus as well as to phytohaemagglutinin. Once a line is established, regardless of whether the cells are B or T, and derived from normal donors or from patients with lymphoproliferative or malignant haemopoietic disorders, it appears capable of living

for ever. Such cell lines may be used for studying human genetics, for forming somatic cell hybrids, and for studying the correlation between the amount of donor DNA incorporated by cells and the extent of the biological effect caused by DNA. These points are discussed briefly, but the main part of the book is concerned with how to maintain healthy cell lines. In this context, it presents a wealth of information on pH control, water preparation, mycoplasma control and cryogenic storage, and the author has succeeded admirably in making the culture techniques and biological assays understandable to anyone interested in establishing and manipulating cultured human lymphoid cell lines.

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Information Section

ARTICLES OF GENERAL INTEREST

MSG—MAINLY REPRODUCTIVE EFFECTS

Monosodium glutamate (MSG) has been suspected of causing brain damage in neonates of a number of species (*Cited in F.C.T.* 1977, **15,** 347). In adult mice that had been given MSG neonatally, hypothalamic lesions giving rise to multiple endocrine dysfunction had been described (*ibid* 1978, **16,** 393). In a more recent review (*ibid* 1979, **17,** 541), the effects of oral doses of MSG on various animal species were described. Further reports of the effects of MSG on the hypothalamus and gonads are now considered.

In order to determine whether the neurotoxic effects of MSG could be attributed to the accumulation of glutamic acid (GA) or sodium or both in the hypothalamus, Airoldi *et al.* (*Toxicology Lett.* 1979, **3,** 121) treated 4-day-old rats, by gavage, either with MSG (in 20% aqueous solution) at a dose level of 2 g/kg body weight, or with an equimolar dose of sodium as 0·68 g NaCl/kg. Adult rats, fasted overnight before treatment, were given 4 g MSG/kg or 1·37 g NaCl/kg. No accumulation of GA or sodium in the hypothalamic arcuate nucleus (HAN) or the lateral thalamus was observed 60 or 180 min after treatment in neonatal rats or 15, 30, 60, 180 or 360 min after treatment in adult rats. GA levels were increased eight-fold in the plasma of infant rats after 60 min, and 11-fold in the plasma of adults after 30 min. In both infants and adults, the sodium concentration in the plasma and HAN, but not in the lateral thalamus, increased after administration of NaCl. In similar studies in guinea-pigs (Airnoldi & Garattini, *ibid* 1979, **4,** 313) there were no increases in GA or sodium levels in the HAN or lateral thalamus of 3-day-old or adult animals given doses of 2 or 4 g MSG/kg, respectively, by gavage. Equimolar doses of NaCl increased the sodium level only in the HAN of neonates (after 180 min). There was a marked increase in plasma GA 60 min after treatment with MSG in both age groups. Thus it seems that the neurotoxicity of MSG cannot be attributed to accumulation of GA or sodium in the hypothalamus.

We have previously reported (*Cited in F.C.T.* 1979, **17,** 541) on studies that have indicated that the route of MSG administration can affect plasma-GA levels. Similar results have been obtained in a more recent study (O'Hara & Takasaki, *Toxicology Lett.* 1979, **4,** 499). Gastric intubation of 10-day-old mice with 0·7 g MSG (as a 10% w/v aqueous solution)/kg body weight raised plasma GA to 1·08 mmol/litre and induced hypothalamic lesions in two out of eight animals. Intubation with 1 g MSG/kg body weight given as a mixture with infant food raised plasma GA to 1·26 mmol/litre and caused no hypothalamic lesions. When MSG was given *ad lib.* at 10 or 15% (w/w) in the diet or at 5% (w/v) in the drinking-water, the plasma GA levels induced in weanling and adult mice were lower than the minimum effective levels for hypothalamic lesions. Such levels were determined to be 1·0–1·3, 3·85 and 6·3 mmol/litre for infant, weanling and adult mice, respectively.

There is evidence that the MSG-induced lesions of the HAN result in disturbances of the endocrine system which lead to reproductive dysfunction (*Cited in F.C.T.* 1977, **15,** 348; *ibid* 1978, **16,** 393). Inhibition of follicle development has been observed in the ovaries of hamsters injected subcutaneously (sc) with MSG as neonates (*ibid* 1977, **15,** 348). Lamperti & Blaha (*Biol. Reprod.* 1979, **21,** 923) carried out a study designed to investigate whether the basis of this inhibition of follicle development was endocrine dysfunction and not a direct effect of MSG on the ovaries. Hamsters were injected sc with 8 g MSG/kg body weight or with saline at 8 days of age. All of the animals given MSG developed lesions in the HAN. When examined at least 2 months after treatment, MSG-treated hamsters had significantly higher progesterone levels in the plasma and ovarian interstitial tissue than did the saline-treated controls, although both groups showed similar 3β-hydroxysteroid dehydrogenase activity. The interstitial cells were enlarged in the animals treated with MSG, and small follicles in the primary and secondary stages were present. In contrast, fresh corpora lutea were present in the controls. Saline-treated animals were ovariectomized and then given ovarian transplants from either MSG-treated or control animals. Following the transplants, all of the animals had consecutive oestrous cycles and levels of luteinizing and follicle-stimulating hormones comparable to those in the controls. Maturation of follicles and formation of corpora lutea occurred in the transplanted ovaries, regardless of their origin. This indicated that the ovaries from the MSG-treated animals were capable of responding to gonadotrophin stimulation and that MSG did not have a direct effect on the function of the ovaries. The same investigators (Lamperti & Blaha, *ibid* 1980, **22,** 687) studied adult hamsters that had been given 8 g MSG/kg sc on days 7 and 8 of life. All of the females given MSG lacked oestrous cycles and had lower uterine and pituitary weights and lower levels of follicle-stimulating hormone in plasma and anterior pituitary glands than did the controls. Their ovaries contained only small follicles. Although 3β-hydroxysteroid dehydrogenase activity in the interstitial cells of the ovary was similar to that in controls, the cells themselves appeared hypertrophied. In all of the males given MSG, the weights of the testes, seminal vesicles and pituitary gland were decreased and plasma follicle-stimulating hormone levels were lower than in the controls. Atrophy of the seminiferous tubules was evident in three out of eight animals. Activity of 3β-hydroxysteroid dehydrogenase in the interstitial cells was reduced. The lesions in the HAN were more severe in females than in males.

Time-related effects of MSG on the reproductive

neuroendocrine axis of the hamster were investigated by Lamperti *et al.* (*Endocrinology* 1980, **106,** 553). Neonatal hamsters were given a single sc injection of 8 g MSG/kg between days 1 and 15 of age. Examination of the HAN 6 hr after the injection showed that the most severe lesions occurred in animals treated on days 7, 8, 9, 10 or 11. The percentage of intact neurons in the HAN was 82% in hamsters treated on day 1, but only 14–19% in those injected between day 7 and day 11. In hamsters treated after day 11 the percentage of intact neurons increased and was 71% in those treated on day 15. In female adult hamsters (at least 60 days old) that had been given a single injection of MSG (8 g/kg) between days 4 and 15 of age, a similar decline in the percentage of intact neurons was seen in those that had been treated between day 4 and day 8. Only 56–60% of the animals that had been treated on day 7 or 8 had oestrous cycles, compared with 75–100% of those that had been treated on other days. Treatment on day 7 or 8 had less effect on oestrous cycles than treatment on days 7 and 8 (Lamperti & Blaha, *Biol. Reprod.* 1980, **22,** 687).

The results of a study by Matsuzawa *et al.* (*Toxicology Lett.* 1979, **4,** 359) provide further evidence that MSG induces abnormalities in reproductive endocrine function in rats when it is injected in large doses, parenterally, early in postnatal life. Female rats that had been treated sc each day on days 2–11 of age with 4 g MSG/kg as a 10% aqueous solution showed severe reproductive endocrine disturbances, such as obesity, mild stunting, precocious puberty, disturbed oestrous cycles and abnormal follicular maturation and ovulation. Females treated in the same way when infants (10–19 days of age) showed far fewer adverse effects. Males were less affected by neonatal MSG treatment than were the females, but showed obesity, mild stunting, decreased gonad weights and increased pituitary concentrations of gonadotrophins. As in the females these effects were diminished when the animals were treated as infants. No abnormalities were observed in either males or females given subneurotoxic doses (0·2 g/kg) neonatally, in those given MSG (0·5 g/kg) orally on days 10–19 of age or in those given MSG at 5% (w/w) in the diet for 10 days after weaning.

Decreased fertility has been observed in male rats treated neonatally with MSG. Bakke *et al.* (*Neuroendocrinology* 1978, **26,** 220) gave MSG sc in progressively increasing doses (2·0–3·5 g/kg) on days 1–5 after birth and as well as reduced fertility found reduced body weights in pups of litters resulting from matings between treated males and control females. Pizzi *et al.* (*Neurobehav. Toxicol.* 1979, **1,** 1) also observed decreased fertility in male rats given sc doses of MSG increasing from 2·2 g/kg on day 2 after birth to 4·4 g/kg on day 11. However they reported significantly increased body weights in the offspring of treated males, and there was a tendency for the litter size to be decreased, although the decrease was not significant. Bakke *et al.* (*loc. cit.*) found no effect of MSG on fertility in females. In both studies the MSG-treated animals showed stunted growth and decreased gonad weight.

Although the majority of evidence points to endocrine dysfunction arising from neonatal treatment with MSG, Olney *et al.* (*Brain Res.* 1976, **112,** 420) reported that a single sc injection of MSG (1 g/kg) induced an acute elevation of serum luteinizing hormone and testosterone levels in adult male rats. Yonetani & Matsuzawa (*Toxicology Lett.* 1978, **1,** 207) further investigated these effects and gave adult male rats *ad lib.* diets containing 4 or 8% MSG (about 2·8 or 5·2 g/kg body weight/day, respectively), and examined them from day 8 after the start of treatment. No significant changes in the circadian fluctuation of serum luteinizing hormone or testosterone occurred. Other rats were given a single sc injection of 1 mg MSG/kg 0·25, 1, 2 or 4 hr before they were killed. The injections were carried out during both the light and dark phases (12 hr light, 12 hr dark). When the injection was given during the light phase, no change in luteinizing hormone level was detected. However, when it was given during the dark phase, MSG treatment reduced the level of luteinizing hormone after 1 hr and increased it after 2 and 4 hr. There was no significant change in luteinizing hormone level in the NaCl-treated (dark phase) controls until 4 hr after the start of treatment, when it rose. In the light phase, the testosterone level was reduced by MSG after 1, 2 and 4 hr; in the dark phase it was significantly decreased only after 1 and 2 hr. In the NaCl-treated controls testosterone showed a decrease only at 4 hr after treatment in the light phase. The MSG-treated groups did not show an initial hormone elevation at 0·25 hr as reported by Olney *et al.* (*loc. cit.*).

Thus, the reproductive effects of MSG given in large doses to neonatal rodents seem well established, but evidence for effects of administration to adult animals is much less well defined. These effects seem to have little relevance to man, a view reflected by the conclusion of the FASEB Select Committee on GRAS substances earlier this year that MSG lacked evidence of hazard at current use levels (*Food Chemical News* 1980, **22** (32), 17).

[P. Cooper—BIBRA]

CELL DIFFERENTIATION—A POSSIBLE POINT OF ATTACK

The survival of complex multicellular organisms such as mammals depends upon the ability of individual cells to carry out specific, specialized functions. Each cell expresses a set of characteristics (its phenotype) which enables it to fulfil its role in the body of the organism. Any inappropriate change in the phenotype of cells is likely to cause some disruption to the correct functioning of tissues and organs. The phenotype of a cell is generally regarded as being controlled by DNA since this is the only information store of the cell that, because it is known to be sufficiently stable and is conserved during cell replication,

can account for the stability of cellular activities. Chemical modification of the primary structure of the DNA of somatic cells (somatic mutation) is therefore one obvious way in which the behaviour of cells might be permanently modified.

That mutation may not be the only way in which permanent phenotypic changes in cells might be effected is indicated by work on normal development and differentiation. The somatic cells of an organism are derived from a single cell and yet during development, a multitude of quite distinct and stable phenotypes appear. Several lines of evidence suggest that despite the great differences in behaviour between cell types, the complement of DNA present in each cell and the primary structure of this DNA remain unchanged. For example, if the nucleus of a differentiated adult frog cell is implanted into an oocyte, it can sometimes give rise to a normal adult organism (Gurdon & Uehlinger, *Nature, Lond.* 1966, **210,** 1240), implying that all the genes required to construct an entire animal have remained intact during differentiation. Similarly, genes that have become inactive during differentiation can be reactivated under certain circumstances. Chicken erythrocytes contain nuclei that are inactive in DNA, RNA and protein synthesis but that can be reactivated to a considerable extent when introduced into the cytoplasm of dividing cells (Ringertz *et al. Proc. natn. Acad. Sci. U.S.A.* 1971, **68,** 3228). Human leucocytes that do not synthesize albumin can be induced to do so by fusing them with hepatoma cells (Darlington *et al. Science, N.Y.* 1973, **185,** 859). In view of these results it seems unlikely that somatic mutation is responsible for the loss or appearance of specific gene activities during differentiation.

The existence of mechanisms that can cause permanent and drastic changes in cellular phenotype without altering the primary structure of DNA cannot seriously be doubted. It can therefore be asked whether the toxic effects of some chemicals might not be explicable in terms of interference with such control mechanisms. In particular, it is important to try to distinguish between phenotypic changes brought about by mutation and those caused by disrupting differentiation. Some of the possible effects that might be expected to result from interference with normal gene regulation are described below.

During embryonic development, patterns of gene expression in different cell types must be closely controlled and co-ordinated. The cells of each developing tissue must change their behaviour with time in a highly integrated manner. Disturbance of gene regulation in any given cell type would be expected to lead not only to aberrant development of the tissue concerned but also to a breakdown in the overall pattern. Even transient perturbation of the response of certain cells could lead to the failure of co-ordinated interactions such as tissue induction. Interference with gene regulation might thus constitute an important mechanism in teratogenicity.

The correct functioning of many tissues is dependent upon the maintenance of a balance between cell renewal (by mitosis) and differentiation (expression of specific function). In the skin, for example, cells are constantly replaced by the multiplication of undifferentiated stem cells while functional integrity is maintained by the differentiation (keratinization) of a fraction of the cells produced. The balance between the two phenotypically different cell types must be maintained by close control of gene expression. Disturbance of this mechanism would result in imbalance e.g. in the skin, hyperplasia or hyperkeratinization could result.

The activity of whole tissues could be disrupted by agents that altered the pattern of gene expression in mature differentiated cells. The effects of hormone agonists or antagonists might be of this type.

Since at least 1829, cancer has been regarded by many as a disturbance of differentiation. This view is still widely held today. In view of the importance of carcinogenesis as a toxicological problem, some of the evidence for this theory will be reviewed here. For full reviews of these topics see Burnet (*Adv. Cancer Res.* 1978, **28,** 1), Coggin & Anderson (*Adv. Cancer Res.* 1974, **19,** 105), Ibsen & Fishman (*Biochem. biophys. Acta* 1979, **560,** 243) and Uriel (*Adv. Cancer Res.* 1979, **29,** 127).

It is a common observation that many tumours tend progressively to lose their differentiated character and to develop a behaviour reminiscent of some aspects of embryonic cells. This "tumour progression" (Foulds, 1969) has been interpreted by some as a form of retrograde differentiation (Uriel, *loc. cit.*).

Many tumours produce substances identifiable as normal products of foetal cells and that are not normally found in adult tissues except in some cases during regeneration or infectious diseases. These 'foetal antigens' are generally the counterparts of common products found in the adult (Uriel, *loc. cit.*).

Some tumours produce normal substances ectopically, suggesting that genes that are normally inactive in the tissue of origin have been re-expressed (Coggin & Anderson, *loc. cit.*).

In many systems, the development of tumours from carcinogen-treated tissues is enhanced by or even dependent upon treatment with tumour promoters. The most potent tumour promoters known, the phorbol diesters, have profound effects on differentiation in many systems (Scribner & Suss, *Int. Rev. exp. Path.* 1978, **19,** 137; Yamasaki, in *Molecular and Cellular Aspects of Carcinogen Screening Tests.* Edited by R. Montesano, H. Bartsch & L. Tomatis. p. 91. IARC, Lyon, 1980).

Teratocarcinomas are malignant tumours that show extensive differentiation into a wide variety of tissues both *in vivo* and *in vitro.* When teratocarcinoma cells are injected into developing blastocysts, they are able to take part in apparently normal development and differentiation. In this form of cancer, abnormal differentiation is clearly not the result of somatic mutation (Mintz & Illmensee, *Proc. natn. Acad. Sci. U.S.A.* 1975, **72,** 3585).

From the above observations, it seems that disturbances of differentiation must be intimately involved in carcinogenesis. However, very strong evidence has also accumulated in favour of the somatic mutation theory of cancer. It may well be that while disturbance of differentiation is the disorder giving rise to malignancy, one of the most important ways in which cellular control mechanisms are disrupted could be by somatic mutations in regulatory genes.

Some cell types in culture can be used to test

chemicals simultaneously for mutagenicity and the ability to induce transformation. When the frequency of specific mutations such as ouabain resistance is compared with the frequency of cell transformation in one carcinogen-treated cell population, the transformation frequency is generally found to be much higher (10 to 1000 times) than the mutation frequency at a known locus (Barrett & Ts'o, *Proc. natn. Acad. Sci. U.S.A.* 1978, **75,** 3297; Landolph & Heidelberger, *ibid* 1979, **76,** 930). If cell transformation is accepted as the counterpart of carcinogenesis then this result casts doubts on the mutational basis of cancer induction.

In recent years, considerable effort has been expended on the development of techniques for the detection of chemically induced genetic damage. In this respect cell culture has proved to be a very powerful technique. Tests for induced chromosomal aberration and point mutation in cultured mammalian cells are widely used in genetic toxicology. The relevance of the results of such tests to the estimation of chemical hazards to human health is still in question. It is argued, however, that chemicals that cause mutation in cultured cells may also cause heritable and somatic mutations in exposed humans. Although direct proof of the importance of somatic mutations is lacking, there is evidence that this mechanism may play a role in carcinogenesis and teratogenesis.

In contrast, the possible involvement of disruption of gene regulation in the toxic effects of chemicals has been largely neglected. One of the main reasons for this neglect is the fact that all the cell systems used in genetic toxicology use cell lines adapted specifically for rapid growth in culture. These lines show behaviour that is in many respects grossly abnormal. Most have abnormal karyotypes and many can be described as transformed. Very few of the cell lines used show any differentiated functions, although little effort is usually made to detect such activities. While cells of this type were ideal for adapting pre-existing microbiological culture and genetic techniques to the study of mammalian cells, such abnormal lines can tell us very little about the effects of chemicals on differentiation and gene regulation.

Any attempt to remedy this situation is hindered by the fact that very few normal cell types will maintain their specialized functions or continue to differentiate in culture. This may be due to the fact that tissue culture methods have been developed mainly for the purpose of supporting and encouraging cell division. However because of the considerable advantages offered by cell culture as a means of investigating cell control mechanisms it is worth considering whether any cell type exists that would be suitable for studying gene regulation.

Many types of cells derived from differentiated tumours will retain their specialized activities in culture. Teratocarcinoma cells are particularly interesting because they continue to differentiate into many tissue cell types. One must, however, have reservations about using cancer cells, which may well have abnormalities of the very biological mechanism under study.

Some cell lines will, under certain circumstances, show differentiation in culture. The mouse 3T3 line, for example, gives rise to fat cells (Green & Kehinde, *Cell* 1975, **5,** 19). Another mouse line, C3H 10T½, can be induced by five-substituted cytosines to develop into at least three distinct cellular phenotypes (Taylor & Jones, *Cell* 1979, **17,** 771). Neither of these lines can be regarded as normal. Both are subtetraploid and neither differentiates in any regular manner under normal circumstances.

Of the many types of normal mammalian cell that might be removed from an animal and cultured over short periods, very few will retain their differentiated characteristics under these circumstances. Of these, myoblasts from developing skeletal muscle show most promise as a differentiating model system. It has been known for many years that rat myoblasts can be grown in culture, cloned and passaged and induced, almost at will, to differentiate into mature muscle fibres (Konigsberg, *Science, N.Y.* 1963, **140,** 1273; Yaffe, *Proc. natn. Acad. Sci. U.S.A.* 1968, **61,** 477). The differentiation process has been studied in some detail and many biochemical markers can be used to demonstrate changes in synthetic activity (see Blau & Epstein, *Cell* 1979, **17,** 95). Of great advantage, however, is the fact that muscle differentiation is visibly obvious. Large, striated, contracting fibres can be seen with the naked eye.

In the course of experiments on myoblasts, a number of workers have observed the effects of chemicals on the differentiation process. For example, some organic solvents, fatty acids, base analogues, steroids and phorbol esters all inhibit muscle differentiation (see Blau & Epstein, *loc. cit.*). The effects on myoblasts of X-irradiation (Friedlander *et al. Devl Biol.* 1978, **66,** 457), 5-azacytidine (Ng *et al. J. Cell Physiol.* 1977, **90,** 361) and *N*-methyl-*N'*-nitro-*N'*-nitrosoguanidine (Loomis, *Proc. natn. Acad. Sci. U.S.A.* 1973, **70,** 425) have been reported. In none of these studies, however, has a quantitative approach been taken and no systematic study of the effects of toxic chemicals has apparently been carried out. The main interest of these workers has been in dissecting the differentiation process rather than assessing the importance of the process as a possible toxicological target.

Since methods already exist for the culture of mammalian myoblasts and for the biochemical characterization of the differentiated muscle fibres, it should be a relatively simple matter to test the effects of a variety of chemicals on the differentiation process.

In an attempt to elucidate the relationship between somatic mutation. transformation and differentiation it would be necessary to adapt existing cytogenetic procedures for use with myoblasts. There seems to be no reason why dividing myoblasts. treated with mutagens, should not be subjected to testing for chromosome damage and mutation by standard methods. Such tests could then be run concurrently with a test of the ability of the treated cells to differentiate. Virus transformation of myoblasts has been reported (Hynes *et al. Devl Biol.* 1976, **48,** 35) and it should, therefore, be possible to test the cells also for chemical transformation.

It would be of particular interest to study the growth kinetics of differentiating cultures to determine whether a definite stem-cell fraction is maintained and whether chemicals can affect the relationship between cell division and progress to terminal differentiation. Differences in sensitivity between

dividing and post-mitotic myoblasts would also be open to study.

In terms of studying teratogens, the myoblast system may not be ideal because it encompasses only the terminal differentiation phase. The biochemical events occurring at this stage may be qualitatively different from those occurring at earlier embryonic stages. It may, therefore, be worthwhile to attempt the isolation and culture of cells of earlier embryos. Such cells might show a greater diversity of developmental potential and chemical sensitivity.

Although the proposition that disruption of gene regulation may be an important basic mechanism in toxic reactions is still largely speculative it is a possibility that should not be ignored. Cell culture and in particular the skeletal myoblast represents a possible starting point for exploratory investigations.

[B. J. Phillips—BIBRA]

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ABSTRACTS AND COMMENTS

FOOD ADDITIVES

The fate of coumarin in the chicken

Cacini, W. & Ritschel, W. A. (1980). The excretion of coumarin and hydroxycoumarins by the avian kidney *in vivo*. *Archs int. Pharmacodyn. Ther.* **243**, 197.

It is well established that the rat metabolizes coumarin quite differently from man (*Cited in F.C.T.* 1979, **17**, 305; Cohen, *Fd Cosmet. Toxicol.* 1979, **17**, 277); in humans 7-hydroxylation of coumarin is the main metabolic pathway, but in rats this occurs to only a limited degree. It is therefore desirable to look for an animal model for studying coumarin that is more closely allied to man. The results of the study cited above suggest that the chicken may provide such a model.

The experiments were carried out on unanaesthetized chickens using the Sperber technique (Cacini & Quebbemann, *J. Pharmac. exp. Ther.* 1978, **207**, 574; Sperber, *Zool. Bidr. Upps.* 1948, **27**, 429). This technique exploits the fact that in birds each kidney is supplied with blood from a separate portal system which receives blood mainly from the veins draining the ipsilateral leg. Therefore a substance infused into a leg vein will pass through the ipsilateral kidney before entering the general circulation and the substance will appear in excess in urine from the infused kidney. Separate collection of urine from each kidney can be carried out because birds have no bladder and each ureter has a separate opening. The apparent tubular excretion fraction (ATEF) is calculated as the ratio of the excess of infused test substance appearing in the infused-side urine/min to the amount infused/min. A marker compound, which is almost completely extracted from peritubular blood in a single pass through the kidney, is infused with the test substance in order to determine the fraction of blood from the infused leg that is routed through the portal system. The ratio of the ATEF to the test substance to that of the marker substance is termed the tubular excretion ratio (TER). Diffusion alone cannot generate a TER $> 0{\cdot}08$. In the study cited above a TER $> 0{\cdot}1$ was considered to be evidence of active tubular excretion.

In the study ^{14}C-labelled coumarin, 7-hydroxycoumarin (7HC) or 4-hydroxycoumarin (4HC) was infused at a rate of 0·5 μmol/min into one leg vein of four chickens. The marker compound was either a [^{3}H]tetraethylammonium salt (5×10^{-4} μmol/min) or *p*-aminohippuric acid (0·4 μmol/min). A series of six to eight consecutive 5-min urine samples were collected, beginning 20 min after the start of infusion. Both 7HC and 4HC were excreted as the corresponding glucuronides (7HCG and 4HCG). Very little conversion to 7HCG occurred when 7HC was incubated with chicken blood, and thus it was concluded that largely unchanged 7HC arrived at the infused kidney and that conjugation occurred intrarenally. Most (84%) of the infused coumarin was excreted as 7HCG, and 8% of the dose was excreted as a conjugate of *o*-hydroxyphenylacetic acid. This suggested that the excretion of coumarin in the chicken is similar to that in man. Comparison of urine from control and infused kidneys indicated that coumarin and 4HC were not excreted by active tubular secretion. The mean TERs for coumarin and 4HC were 0·048 and 0·056%, respectively. In contrast, the mean TER for 7HC was 31%. This evidence for an active excretory process for 7HC was supported by the results of a further experiment in which probenecid (a potent inhibitor of organic anion transport) was found to reduce the TER values significantly for both 7HC and *p*-aminohippuric acid (used as a reference organic anion). The results of earlier studies (*Cited in F.C.T.* 1979, **17**, 305) have indicated that in man active tubular excretion of 7HCG is involved in the body clearance of coumarin. It seems therefore that there is a close similarity between the coumarin excretion patterns in man and the chicken.

Back to BHT basics

Nakagawa, Y., Hiraga, K. & Suga, T. (1980). Biological fate of butylated hydroxytoluene (BHT)—binding of BHT to nucleic acid *in vivo*. *Biochem. Pharmac.* **29**, 1304.

A large financial investment in the study of butylated hydroxytoluene (BHT) has resulted in limited knowledge of its histological effects in a single species, the rat, but little else. Attempts are now being made to uncover the biochemical basis of its biological activity, such an understanding being prerequisite for a confident verdict on safety-in-use. The authors of the study cited above have previously shown that BHT can be metabolized by a cytochrome *P*-450-linked monooxygenase enzyme system in the liver of rats to compounds that are capable of binding to cellular macromolecules (Nakagawa *et al. Chem. pharm. Bull., Tokyo* 1979, **27**, 480). Their latest study further defines BHT's binding characteristics.

The distribution of bound radioactivity in the liver was determined in male Wistar rats that had been given [^{14}C]BHT by gavage at a dose of 5 mg/rat. The amount of the label bound to the protein fraction of the liver reached a maximum 6 hr after dosing. However, that associated with the nucleic acids was much more stable, and remained at a constant level for 1 wk. During this time the ratio of the radioactivity in the nucleic acids to that bound to the nucleic acids and protein combined had increased from 5 to 50%. Within the nucleic acid fraction itself, the persistence of radioactivity in RNA was far higher than in DNA. By day 7 for example, the extent of binding to the RNA was some 18 times that to the DNA (and 35

times the level bound to protein). The radioactive BHT metabolite(s) were thought to be bound to the macromolecules covalently since the radioactive material(s) could not be removed by exhaustive extraction with methanol–ether (1:1, v/v) or with ether.

Pretreatment of the rats with phenobarbital (five daily intraperitoneal injections of 80 mg/kg) before intubation with BHT (this time at 50 mg/kg) produced a general increase in the binding of radiolabelled metabolites to the liver macromolecules. This increase corresponded to the previously reported increase in BHT-oxidase activity in response to phenobarbital treatment (Nakagawa *et al. loc. cit.*). In their future work the investigators will be attempting to identify the bound BHT metabolite(s). Interpretation of the present findings must await the results of these additional studies.

Caffeine teratology—confusing behaviour?

Sobotka, T. J., Spaid, S. L. & Brodie, R. E. (1979). Neurobehavioral teratology of caffeine exposure in rats. *Neurotoxicology* **1**, 403.

It is sometimes said that nothing more than the puritan ethic underlies attempts to persuade the general populous to abstain from their stimulating cups of caffeine. However, just a glance at the (long) list of studies that the FDA feel would be needed if caffeine is to maintain its position as a permitted food additive (*Federal Register* 1980, **45**, 69817) suggests that the oft-quoted suspicions might have some scientific basis since doubts seem to remain on toxicity, teratogenicity and carcinogenicity. A study (cited above), conducted at the FDA's own laboratories, of the developmental and behavioural profiles of rats treated perinatally with caffeine illustrates a seemingly common feature of toxicological studies involving caffeine—that they invariably appear to generate interesting but uninterpretable results!

Caffeine adulteration of the drinking water of three groups of pregnant Sprague–Dawley rats, was started on day 7 of gestation and continued throughout pregnancy and lactation. The concentrations of the caffeine solutions were 0 (control), 0·0125, 0·025 and 0·5% and these provided doses to the dams of about 0, 23, 49 and 92 mg/kg/day, respectively, throughout pregnancy. The daily doses were increased by about 50% during lactation because of a non-treatment-related increase in fluid intake.

The litters were culled to 10 pups/litter on day 1 in favour of male pups. During the first 21 days of life, developmental profiles of the offspring were made and neuromotor behaviour on one male and one female of each litter was measured on every third day. No consistent statistically-significant effects of litter size or sex distribution were seen amongst the groups and although there was a slight increase in post-natal mortality in the caffeine-treated animals, this was not dose-related. A similar treatment-related (but not dose-related) slight delay in eye opening and slight, transient reductions in post-natal growth were noted. Neither mobility nor the surface-righting reflex was affected by caffeine exposure.

At 21 days of age, the female pups were discarded and the male pups were weaned and housed singly. The males that had not been used for developmental reflex testing were assigned to one of three groups. Rats in one group were killed at 23 days of age for neurochemical analysis. Another group was subjected to a test for general motor activity and to two studies aimed at assessing the animals' response to stress, the total programme occupying days 28 to 50; the caffeine-exposed and control animals exhibited similar behavioural patterns. The third group was used in open-field testing to assess exploratory activity at day 35, and from day 70 to 160 was checked for 'operant conditioning'. The latter test was a means of assessing higher nervous system function and involved the training of the rats to press a selected lever to receive a food reward. Exploratory activity was increased in the caffeine-treated groups compared with the controls. In the operant conditioning tests the performance of the caffeine-treated rats was relatively poor in the initial (training) part of the testing programme but the superiority of the controls was not maintained. Indeed the caffeine-exposed rats had a facilitated performance in the later tests (in which food rewards were not given every time the rats pressed the correct lever). They also exhibited a certain discrimination in their actions (comparable to habit formation) in the absence of a food reward, whereas this effect was not seen in the controls. The fact that these behavioural changes were seen long after caffeine treatment had finished indicated that perinatal exposure may have produced permanent neurological damage. In the present experiment there was only a limited biochemical study of the weanling rats' brains, and none of the parameters measured, which included DNA, protein, cholesterol, acetylcholinesterase and norepinephrine contents, were any significant treatment-related effects seen. Obviously more detailed study of the brains of similarly treated animals may pay dividends.

[The results of a conventional teratology study, recently announced by the FDA, indicated that caffeine can cause physical malformations in rats at relatively low dose levels (*Food Chemical News* 1980, **22** (26), 35). The possibility that the rat may be uniquely ill-equipped to cope with caffeine stress has still to be excluded. Many of the doubts over the safety of caffeine arise from rat experiments and some effort aimed at checking the metabolic similarity of the rat to man seems to deserve a higher priority than further detailed teratogenicity studies in rats.]

NATURAL PRODUCTS

Salted fish: a Hong Kong hazard?

Fong, L. Y. Y., Ho, J. H. C. & Huang, D. P. (1979). Preserved foods as possible cancer hazards: WA rats fed salted fish have mutagenic urine. *Int. J. Cancer* **23**, 542.

Salted fish incubated with sodium nitrite has been shown to be mutagenic (*Cited in F.C.T.* 1978, **16**, 393), and *N*-nitrosodimethylamine (NDMA) has been detected in samples of salted fish from Hong Kong (Fong & Chan, *Fd Cosmet. Toxicol.* 1973, **11**, 841; *idem, ibid* 1976, **14**, 95). A further study of seafoods traditionally eaten in southern China has also indicated the presence of mutagens.

Two samples of dried shrimps and samples of four different species of salted fish were minced and extracted with dimethylsulphoxide and the extracts were tested in an Ames test using *Salmonella typhimurium* strains TA98 and TA100. All of the extracts were mutagenic to both strains, their effect usually being enhanced by the addition of rat-liver postmitochondrial supernatant (S-9) mix. The mutagenic activity in the urine of rats fed steamed fish for 72 hr was greater than that in the urine of control rats fed salted rat chow. The mutagenic activity in the urine of rats fed steamed salted fish 5 days/wk for 5 months decreased markedly when the rats were given a diet of salted rat chow instead. The addition of S-9 mix increased the mutagenic activity of the urine in strain TA98.

The presence of mutagens, and particularly of volatile *N*-nitrosamines in salted fish has been suggested as one aetiological factor in the development of nasopharyngeal cancer (Geser *et al.* In *Nasopharyngeal Carcinoma: Etiology and Control.* Edited by G. de-Thé & Y. Ito. p. 213. IARC, Lyon, 1978; Anderson *et al. ibid* p. 231).

[In a paper published recently (*Fd Cosmet. Toxicol.* 1981, **19**, 167) Huang *et al.* have confirmed the presence of volatile *N*-nitrosamines (mainly NDMA) in uncooked salted fish and have also found that nitrosamines are formed in the fish during cooking.]

Perinatal safrole exposure

Vesselinovitch, S. D., Rao, K. V. N. & Mihailovich, N. (1979). Transplacental and lactational carcinogenesis by safrole. *Cancer Res.* **39**, 4378.

Safrole is the major flavouring constituent of the root bark of the sassafras tree. It also occurs in various amounts in a variety of spices and essential oils including black pepper, mace, star anise oil, cinnamon leaf oil, cocoa and parsley (Buchanan, *J. Fd Safety* 1978, **1**, 225). In the past, sassafras and its essential oil or synthetically manufactured safrole have been used as flavourings and in soaps and perfumery. Safrole's hepatotoxicity and ability to induce liver tumours are well documented (*Cited in F.C.T.* 1981, **19**, 130). However the study cited above appears to be the first investigation into the perinatal carcinogenicity of safrole and the results indicate that safrole and/or its metabolites can be transferred to perinatal mice across the placenta or *via* the mothers' milk and have tumorigenic affects.

Groups of pregnant or lactating mice were treated with doses of 120 mg safrole/kg body weight which were administered by gastric intubation at 2-day intervals. The pregnant mice were given four doses, starting on day 12 of gestation, and the first of the 12 treatments during lactation was adminstered on the day of delivery. Starting at 28 days of age offspring of untreated mothers were given 120 mg safrole/kg body weight twice weekly for 90 wk. Two additional groups of offspring were given combined treatments; one was exposed during gestation and *via* the mothers' milk and the other was exposed pre- and postnatally and for 90 wk after weaning. Fortnightly weighing and inspection of the 60–80 offspring of each sex in each study group was followed by complete autopsy and histological examination of tumours and lung sections from all animals, whether they were killed when moribund or at the end of the experiment when they were 94 wk old.

The mice exposed to safrole or its metabolites prenatally and/or *via* the mothers' milk had a high survival rate (at least 90%) at 92 wk, but those subjected to direct intubation beginning at the time of weaning (with or without prenatal treatment) had lower rates of survival (about 50% for males and 30% for females). The vast majority (96·8%) of tumours were identified only after the offspring were 60 wk old. Transplacental treatment with safrole did not induce liver tumours. However, liver tumours developed in 33·7% of the males and 2·5% of the females exposed *via* the milk, the sex difference possibly relating to a lack of metabolic competence in the females. A similar incidence of liver tumours (in 32·4% of males and 1·4% of females) was observed in the mice exposed during gestation and *via* the milk. In contrast to these results it was mainly the females (47·8%) rather than the males (8·3%) that developed liver tumours in the post-weaning treatment group. In mice exposed prenatally, in infancy and after weaning the incidence of liver tumours was similar in both sexes (males, 46·3%; females, 59·7%). The results indicated strong modifying effects of sex and of age at treatment on liver carcinogenesis. A total of 44% and 74% of the liver tumours found in males and females, respectively, were classified as hepatocellular carcinomas. The overall rate for pulmonary metastases was higher in females (39%) than males (24%).

Renal epithelial tumours occurred in females that were exposed transplacentally (with or without additional treatment by other routes). Although the incidence of these tumours was low (about 7%) they were not seen in controls, in females exposed *via* the milk and/or during weaning, or in males. These results underlined the effects of age and sex on the carcinogenicity of safrole.

[Some earlier studies have also indicated that adult female mice are more susceptible than males to the carcinogenic effects of orally adminstered safrole and its metabolites (Innes *et al. J. natn. Cancer Inst.* 1969, **42**, 1101; Wislocki *et al. Cancer Res.* 1977, **37**, 1883). However the reverse seems to be true for safrole given subcutaneously to mice (*Cited in F.C.T.* 1974, **12**, 417).]

OCCUPATIONAL HEALTH

1,2-Dibromoethane at work

Ott, M. G., Scharnweber, H. C. & Langner, R. R. (1980). Mortality experience of 161 employees exposed to ethylene dibromide in two production units. *Br. J. ind. Med.* **37**, 163.

The agricultural fumigant ethylene dibromide (dibromoethane; DBE) is carcinogenic in laboratory animals (*Cited in F.C.T.* 1975, **13**, 144; *Federal Register* 1978, **43**, 52775), Using data derived from such a study in a predictive one-hit carcinogen model the Carcinogen Assessment Group of the EPA estimated that an almost 100% lifetime incidence of cancer would be expected in workers exposed to 0·4 ppm DBE for 40 yr (cited by Ramsey *et al. Toxic. appl. Pharmac.* **47**, 411). However, the validity of this model has been questioned (Ramsey *et al. loc. cit.*) in the light of the epidemiological investigations described in the paper cited above which suggest that this assessment may be grossly exaggerated.

Information derived from existing records, discussions with past employees and social security records were used to assess the exposure to DBE and the mortality (with special reference to cancer and respiratory disease) of 99 workers at a DBE manufacturing plant that was operational from 1942–1969 and of 62 in another that operated from the mid-1920s until 1976. Although it was known that replacement of equipment in the 1960s considerably reduced worker exposure levels, quantitative data were not available for the first plant. No other organic bromide products were manufactured there. Analysis of the information that was collected showed that the two deaths from malignant neoplasms that occurred at this unit were less than the number expected (3·6) on the basis of data on US white male mortality. The other major cause of deaths was cardiovascular disease (11 deaths, 9·1 expected). In the second plant workers involved in the operation of reactors and stills were routinely exposed to levels of DBE of up to 110 ppm. Three episodes of acute toxicity (known to be induced at about 75 ppm and above) were reported between 1954 and 1970. The mean serum bromide concentrations measured between 1957 and 1970 ranged from 1 to 17 mg/100 ml (mean 6·5 mg/100 ml) and were above normal background levels (up to 5 mg/100 ml). However the serum bromide concentrations did not specifically reflect DBE exposure since other bromine-containing chemicals were manufactured at the plant. Arsenicals were also produced, and since previous work has indicated that these may increase the risk of respiratory malignancies, the five workers exposed to them were excluded from further analyses. However, it is interesting to note that three deaths (two from respiratory cancer) occurred in this group compared with 0·6 expected. Three deaths among workers not exposed to arsenicals were caused by diseases of the respiratory system compared with 1·3 expected, but there was no apparent connection with DBE exposure. Five deaths from malignant neoplasms were observed compared with 2·2 expected. Examination of mortality in relation to duration of exposure indicated that although not statistically significant there appeared to be an increase in cancer mortality among employees with more than 6 yr exposure, but no specific target site was identified.

These findings, which neither establish nor rule out the possibility that DBE is a carcinogen in man, should be regarded cautiously owing to the limitations of the data. These limitations include the size of the study populations, the variety of toxic agents to which the individuals in one of the plants were exposed and the lack of data on the smoking habits of the workers. However, the need for continued study of this group and further investigations throughout the industry is evident.

No evidence of lung damage by PVC dusts

Chivers, C. P., Lawrence-Jones, C. & Paddle, G. M. (1980). Lung function in workers exposed to polyvinyl chloride dust. *Br. J. ind. Med.* **37**, 147.

Studies to assess the degree of hazard arising from the inhalation of polyvinyl chloride (PVC) dusts have yielded inconclusive results. One of two PVC dust samples was found to be strongly haemolytic *in vitro*, but its activity was attributed to some readily soluble agent on the surface of the particles (*Cited in F.C.T.* 1976, **14**, 158). The toxicity to rat peritoneal macrophages *in vitro* of particles of two PVC emulsion polymers was almost certainly due to the presence of an alkylbenzene sulphonate emulsifier. However, when the particles of one of these emulsion polymers was injected into rats in single intraperitoneal or intratracheal doses, there was no evidence of progressive fibrosis (*ibid* 1981, **19**, 277). On the other hand, lung damage was found in rats and guinea-pigs after 2–7 months continuous exposure to PVC dust in a bagging plant (Frongia *et al. Medna Lav.* 1974, **65**, 321) and there have been isolated reports of pneumoconiosis in PVC workers (e.g. Arnaud *et al. Thorax* 1978, **33**, 191; Szende *et al. Medna Lav.* 1970, **61**, 433). In view of this conflicting evidence, the large-scale study by the authors cited above of lung function in workers exposed to PVC dust is particularly welcome.

The 509 male workers who took part in the study were employed in a factory in which PVC-coated fabrics and wall-coverings were manufactured. Of these workers, 112 were exposed only to non-chlorinated solvents, 104 were exposed only to PVC dust and 293 were exposed to both solvents and dust. The study was carried out in 1977. Air sampling was carried out in the PVC mixing areas in 1978 using personal monitoring devices. The mean respirable fraction of PVC dust was found to range from 0·2 mg/m^3 in a well ventilated mixing room and in the bag-disposal area to 11·5 mg/m^3 in a non-ventilated loading area. Among the dust-exposed group 43% of the men had been exposed for less than 5 yr, 27% for 5–9 yr, 19% for 10–14 yr and 11% for 15 yr or more. In the group exposed to dust and solvents, 38% had been exposed for less than 5 yr, 28% for 5–9 yr, 20% for 10–14 yr and 14% for 15 yr or more. Since there was not a large enough group of workers in the factory who were unexposed to dust or solvents, the solvents-exposed workers were used as controls. Of these

workers, 38% had been exposed to solvents for less than 5 yr, 26% for 5–9 yr, 16% for 10–14 yr and 20% for 15 yr or more.

The workers completed a questionnaire on respiratory function and measurements were made of forced expiratory volume (FEV) and forced vital capacity (FVC). The data were analysed taking into account the height, age and smoking habits of the workers and the effects of the degree and duration of exposure to PVC dust were assessed.

The mean height and age of the men in each exposure group were similar and there was no significant variation in lung function between the three groups. Neither did duration of exposure to PVC have any effect on lung function when the effects of age, height and smoking habits were considered. However, irrespective of exposure group, the FEV and FVC were reduced in smokers in comparison with non-smokers, the reductions being greatest in heavy smokers (those who smoked or had only recently stopped smoking 15 or more cigarettes per day). When smoking and exposure effects were considered separately it was shown that there was no evidence of a deleterious effect of the dust in any of the smoking groups. It was concluded that there was no evidence that exposure to PVC dust over a prolonged period had caused any lung damage among workers in the plant.

Life is also harmful to your skin

Rycroft, R. J. G. (1980). Occupational dermatoses in perspective. *Lancet* **II**, 24.

Amidst the popular concern caused by newly discovered carcinogens, teratogens and mutagens, it is sometimes forgotten that many of the toxicological problems of the workplace are just skin deep, involving as they do, skin irritancy and sensitization. An investigation by the Employment Medical Advisory Service (EMAS) of the laboratory staff of an animal virus research institute who were suffering from a possibly occupational epidemic of skin diseases, illustrates the careful consideration needed before specific work practices are incriminated.

The laboratory work necessitated frequent whole-body showers and (for men at least) hair washing. As an antiviral measure, there was regular washing of the exposed skin of the hands and protective clothes with antiviral disinfectants such as 4% washing soda or 0·2% citric acid or (as a protective measure against the resistant swine-vesicular-disease virus) a mixture of iodine, phosphoric acid, sulphuric acid and non-ionic surfactant. Technicians involved with tissue culture also had occasional accidental contact of their hands with 4% formaldehyde solution. The ventilation system operating in the laboratory resulted in a relative humidity as low as 35%. During 1979, 35 of the 160 laboratory employees had some kind of skin problem. However, after careful clinical examination, judicious patch-testing and detailed examination of their work areas, the EMAS dermatologist considered that only ten of these cases of dermatitis were likely to have been a direct result of the workplace. These comprised six cases of simple irritancy, either to washing soda, the iodine mixture, citric acid or Dettol, two cases of asteatotic eczema probably due to repeated soap-and-water showering together with the low humidity, and two cases of allergic contact dermatitis. One was a result of formaldehyde sensitization, the other of thiuram sensitization arising from the routine wearing of rubber gloves. Thirteen of the original complaints were thought to have been due to non-eczematous dermatoses which were not significantly exacerbated by occupation and one was a case of non-occupational allergic contact dermatitis. In addition, 11 subjects were diagnosed clinically as having various types of endogenous eczema; psoriasis, for example, was present in a total of 3–4% of all employees. Rycroft therefore concluded that while genuine outbreaks of occupational dermatoses from a common cause sometimes affect 10–15% of a workforce, a similar prevalence of skin disease may result from non-occupational causes.

The present report also demonstrated that most conditions could be managed by improving work practices. The routine use of emollient creams after showering, a reduction in the concentration of shampoos, and more care in the preparation and use of the disinfectant solutions resulted in a marked improvement in the majority of subjects who had occupationally induced dermatitis and no job changes were necessary.

ENVIRONMENTAL CONTAMINANTS

The rise and fall of kidney cadmium

Travis, C. C. & Haddock, A. G. (1980). Interpretation of the observed age-dependency of cadmium body burdens in man. *Envir. Res.* **22**, 46.

Cadmium is a non-essential element which accumulates in the body, particularly in the liver and kidneys. It is generally agreed that the available cadmium in the environment is increasing, indeed Kjellström *et al.* (*Archs envir. Hlth* 1975, **30**, 321) found that the level of cadmium present in Swedish wheat has tended to increase almost linearly from 1916 to 1972. The kidney is a major target of cadmium toxicity (*Cited in F.C.T.* 1979, **17**, 84). The results of human autopsy studies show that the cadmium concentration in the kidney increases steadily with age until about age 45 yr when a considerable decline in kidney cadmium concentration begins (Schroeder & Balassa, *J. chron. Dis.* 1961, **14**, 236). The authors of the paper cited above have used a one-compartment model of the human body to investigate the relative importance of various factors in explaining these age-dependent variations in the cadmium concentrations of the human renal cortex.

Initially the contribution of food to the cadmium body burden was assumed to be a constant 0·02 μg Cd/cal, 5% of which was absorbed, and an age-depen-

dent calorie intake was determined on the basis of FAO data. Although the contributions of both air and water to the body burden vary enormously, it was assumed that they could both be considered to be negligible for the population as a whole. To determine the contribution of smoking it was assumed that 0·2 μg Cd was inhaled from each cigarette, and that 25% of this was absorbed through the lungs; the 1966 USA data for average cigarette consumption/day at a range of ages were used. The contribution of smoking was weighted according to the proportion of the population (by age group) who smoke. The authors assumed that one third of the body burden of cadmium was located in the kidneys. They used a widely recognized one-compartment model to compute cadmium in the renal cortex as a function of age for various excretion rates but the shape of the resultant curve did not correspond with the autopsy finding of a considerable decline in body burden above 45 yr of age. Although smoking has been shown to double the cadmium body burden of smokers (*Cited in F.C.T.* 1980, **18**, 552) it did not greatly alter the shape of the curve when included in the model. It was noted that the age of peak kidney burden was related to the magnitude of the excretion rate constant. However, using the model, a peak kidney burden at age 45–50 yr was found to imply a biological half-life that was much lower than the actual figure for man. The authors thus concluded that one or both of their assumptions of a constant cadmium contribution from food and an age-independent excretion rate was wrong. They then considered the possibility that older subjects had not been exposed to present day high levels of cadmium in the environment. They thus incorporated a linear increase in cadmium levels in food into the model. The resulting curves showed that the historical increase in environmental levels of cadmium did not account for the magnitude of the decline in the body burden of cadmium in older age groups.

There is evidence that the rate of cadmium excretion increases with age but most of the increase can be accounted for by the age-dependent increase in cadmium body burden. There is no direct evidence that the increase in cadmium excretion results from the age-related functional and morphological changes that take place in the kidney. However, it has been demonstrated that loss of nephrons with increasing age is a gradual process and that other morphological changes are progressive. The authors therefore assumed that there was a linear age-dependent increase in the cadmium excretion rate. Combining this with the incorporation of a linear increase in cadmium levels in food and including the effects of smoking they found that their model predicted the results for renal cadmium levels from autopsies quite well. On the basis of the modified model the age-dependent biological half-life of cadmium in the renal cortex of man was found to decrease from 35 yr at birth to 11 yr at 80 yr of age.

Lead into monkey business

Bushnell, P. J. & Bowman, R. E. (1979). Effects of chronic lead ingestion on social development in infant rhesus monkeys. *Neurobehav. Toxicol.* **1**, 207.

Behavioural abnormalities have been linked with lead poisoning in children (Chisholm *Pediat. Clins N. Am.* 1970, **17**, 591). The existence of behavioural problems in children exposed to low levels of lead who show no overt signs of toxicity is more controversial. Fifty-four children with a history of pica and elevated body burdens of lead were identified by de la Burdé & Choate (*J. Pediat.* 1975, **87**, 638). Nineteen of these children were described as hyperactive, impulsive, having frequent temper tantrums and being aggressive and inconsiderate towards their peers. Only five control children, matched for age and socio-economic background, had these characteristics. Needleman *et al.* (*New Engl. J. Med.* 1979, **300**, 689) reported dose-related increases in the frequency of 'nonadaptive classroom behaviour' such as distractability, hyperactivity and inability to follow directions or sequences with increasing dentine lead concentrations. However, in its recent report entitled *Lead and Health* (HMSO, London, 1980; pp. 129, £4.50) the DHSS Working Party on Lead in the Environment concluded that studies to date are ambiguous or inconclusive. In the paper cited above the authors describe four experiments in which they investigated the effects of lead on the social behaviour of infant rhesus monkeys.

In the first two experiments groups of three or four monkeys were given lead acetate in milk from 3 wk of age in doses sufficient to increase blood-lead levels to about 30 or 60 μg/100 ml for 1 yr. No treatment-related differences in physical growth, development, food intake or body weight were observed but there was a 10% reduction in haemocrit values for the first 6 months in the high-dose group. Behavioural observations showed that the frequency of 'rough-and-tumble' play was decreased and the tendency of the monkeys to cling to one another was increased. When the monkeys were moved to a different play area at 21 wk of age rough-and-tumble play was greatly suppressed in all groups but recovered rapidly only in the control group, the treated groups taking much longer to return to base levels. The frequencies of locomotion and of physical contact (other than sexual, grooming, clinging or rough-and tumble play contact) with other monkeys (termed 'initiated social explore') also decreased for longer after the move in treated animals than in the controls. The decrease in the frequency of rough-and-tumble play was much less marked when the monkeys were observed in a group that also contained the controls. It is suggested that this may be because the treated monkeys responded to play solicitations from others but were disinclined to initiate play themselves.

In a further experiment (3), groups of four monkeys were exposed chronically to lead acetate at a dose sufficient to maintain blood-lead levels at 80 ± 10 μg/100 ml, or were given a higher dose of lead acetate at 5 and 6 wk of age to give a transient blood-lead level of 300 μg/100 ml. A third group was given a combined treatment. No effects on physical growth and development were observed; haematocrits were reduced by 12–15% in both groups given an acute dose during the period of high blood-lead levels, but from wk 12 to 52 all haematocrits were normal. The frequency of rough-and-tumble play and of approaching other monkeys decreased in the chronically exposed group,

and the development of 'initiated social explore' was suppressed. A change in the play environment induced similar changes to those observed in the first experiment, but to a much lesser extent, probably because the move took place when the monkeys were older (41 wk). The effects in monkeys dosed both chronically and acutely resembled those of the animals only chronically exposed, but were generally less severe. In contrast the acute, transient exposure to lead had no observable effect on the monkeys' behaviour until after the move to a different play area when a dramatic reduction in rough-and-tumble play occurred. At the time of the move these monkeys had a blood-lead level of 10 μg/100 ml and it seemed therefore that latent neurobehavioural damage had occurred that was only expressed under the stress caused by the move.

In the fourth study groups of six female monkeys were given 0, 3, 6 or 9 mg lead/kg body weight/day, in the drinking water, for at least 3 months before mating and throughout pregnancy. Six of the infants from control mothers and ten from treated mothers were separated from the mothers when 2 days old and were observed for 1 yr (despite the differences in lead dose, dams of the three treatment groups could not be distinguished on the basis of blood-lead levels). No behavioural differences were seen between the treated and control infants.

The results of these experiments indicate that postnatal intoxication with inorganic lead, at doses that do not produce signs of overt toxicity, does affect the social development of infant rhesus monkeys. The most notable effect was the reduction in social play in chronically exposed monkeys. The critical factor acting to suppress social play appeared to be dietary lead levels and not blood-lead levels. The evidence for this comes from three of the results of these studies. Normal development of social play occurred in monkeys given a single dose of lead (experiment 3) even though these animals had blood-lead levels (27 μg/100 ml) similar to those at which behavioural abnormalities occurred in the chronically exposed animals in the first two experiments (30 μg/100 ml). Secondly, in experiment 3, the group exposed both acutely and chronically had higher overall blood-lead levels and a higher total lead dose than the chronically exposed group during wk 1–40, but the latter group had to be given higher lead doses during the period (wk 13–40) of data acquisition in the first play area in order to maintain blood-lead at target levels (80 μg/100 ml). The severity of behavioural disturbances was greatest in the chronically exposed animals on the higher daily dose. Lastly, *in utero* exposure to lead had no observable effects on social behaviour.

It seems that low-level lead intoxication in monkeys tends to make them withdrawn and submissive and this contrasts with some of the reports of effects in children. It is suggested that these differences might be explained by a child's frustration at poor performances and failure resulting from impaired learning ability. Such frustration may not occur in the laboratory-reared monkey under the conditions of testing for behavioural responses.

[This study is of interest since it concerns blood-lead levels (35–80 μg/ml) at which effects in children are disputed.]

Carcinogenicity studies on VDC and chloroprene

Ponomarkov, V. & Tomatis, L. (1980). Long-term testing on vinylidene chloride and chloroprene for carcinogenicity in rats. *Oncology* **37**, 136.

Vinylidene chloride (1,1-dichloroethylene; VDC) is widely used in the production of flexible co-polymers for food packaging. VDC has been reported to cause kidney tumours in male mice (Maltoni *et al. Medna Lav.* 1977, **68**, 241). However the hazard to consumers presented by VDC packaging does not appear to be significant. A working party established to ascertain the potential for such exposure concluded in its recent report that the maximum possible daily intake of VDC from food would be no more than 1 μg/person/day (*Survey of Vinylidene Chloride Levels in Food Contact Materials and in Foods*; MAFF Food Surveillance Paper No. 3; HMSO, London, 1980; pp. iv + 19, £2.25). Furthermore, in the study cited above only limited evidence was found of the carcinogenicity of VDC in rats.

About 20 female BD IV rats were given a single intra-gastric dose of 150 mg VDC/kg body weight on day 17 of pregnancy and their offspring (89 males and 90 females) were given weekly doses of 50 mg VDC/kg from weaning until death. Control rats were treated with olive oil, the surviving rats were killed after 120 wk. The litter sizes and survival rates of VDC-treated rats were similar to those of the controls. Liver tumours in both sexes and meningeal tumours in males were more frequent in the treated than in the control animals, but there was no significant difference between the two groups in the total number of tumour-bearing animals.

These results, which provide limited evidence of the carcinogenicity of VDC, are similar to those of previous studies. A possible (not dose related) increase in mammary tumours was observed in rats inhaling VDC (Maltoni *et al. loc. cit.*). Lee *et al.* (*J. Toxicol. envir. Hlth* 1978, **4**, 15) reported the induction of haemangiosarcomas in the mesenteric lymph node or the subcutaneous tissue in two rats that inhaled 55 ppm VDC. However Rampy *et al.* (*Envir. Hlth Perspect.* 1977, **21**, 33) found no increase in tumour incidence in rats after oral administration or inhalation exposure.

Ponomarkov & Tomatis (*loc. cit.*) also investigated the carcinogenicity of chloroprene, which has been used for many years in the production of synthetic rubber. Chloroprene was mutagenic in an Ames test (*Cited in F.C.T.* 1976, **14**, 220). However, a study of occupationally exposed workers (Pell, *J. occup. Med.* 1978, **20**, 21) indicated that chloroprene exposure did not increase the risk of lung cancer. In the present study female BD IV rats were given 100 mg chloroprene/kg body weight by gastric intubation on day 17 of pregnancy and the offspring (81 males and 64 females) were given weekly doses of 50 mg chloroprene/kg from weaning until death. The results gave no evidence of carcinogenicity.

Benzene and vinylidene fluoride carcinogenicity

Maltoni, C. & Scarnato, C. (1979). First experimental demonstration of the carcinogenic effects of benzene. Long-term bioassays on Sprague–Dawley rats by oral administration. *Medna Lav.* **5**, 352.

Maltoni, C. & Tovoli, D. (1979). First experimental evidence of the carcinogenic effects of vinylidene fluoride. Long-term bioassays on Sprague–Dawley rats by oral administration. *Medna Lav.* **5,** 363.

Both vinylidene fluoride (VDF) and benzene are widely used—VDF in polymers and in the synthesis of waxes, and benzene in petrol production, as a chemical intermediate in the production of a vast array of industrial compounds and as a solvent in paints and rubbers. About 2000 tons of VDF are estimated to be produced per annum but this is relatively minor compared with the 15 million tons of benzene prepared around the world every year. Despite this extensive use, many toxicological data are lacking on both of these compounds. No data on the chronic toxicity of VDF have been available until the present study and although the long-term effects of benzene have been well described in humans (*Cited in F.C.T.* 1977, **15,** 652; *ibid* 1978, **16,** 299) few experimental carcinogenicity bioassays have been undertaken. The IARC concluded that the subcutaneous injection and skin application tests undertaken in mice did "not permit the conclusion that carcinogenic activity had been demonstrated" (IARC Monographs on the Evaluation of the Carcinogenic Risk of Chemicals to Humans, Vol. 7, p. 203. IARC, Lyon, 1974). Later injection studies also failed to demonstrate carcinogenicity in mice (*Cited in F.C.T.* 1976, **14,** 157).

Maltoni *et al.* (cited above) gave benzene or VDF, dissolved in olive oil to groups of about 30 13-wk-old Sprague–Dawley rats by gastric intubation. Two doses were tested in each case; in the benzene study, 50 and 250 mg/kg body weight were used while in the VDF study the doses were 4·12 and 8·25 mg/kg. Dosing was undertaken once daily, 4–5 days/wk for 52 wk. The animals were then kept untreated until spontaneous death. The VDF study ended at 141 wk and the benzene study at 144 wk. Gross pathological and histophathological changes were noted at autopsy.

In the VDF study, nearly 9% of the animals had tumours of the adipose tissue (lipomas and liposarcomas) compared with 4% of the control males and none of the control females and with an incidence of 0·5% in the general Sprague–Dawley population used at the study centre. In the female rats treated with benzene there was an increased incidence of carcinomas of the Zymbal gland and this incidence seemed to be dose-dependent (7% at the lower dose level and 25% at the higher level). No Zymbal gland carcinomas occurred in male rats or in the controls. In addition, there were dose-related increases in haemolymphoreticular neoplasms (leukaemias) and mammary carcinomas, and at the higher dose level tumours rarely seen in the rats of this colony (skin carcinomas, angiosarcomas and hepatomas) were also observed.

Both of these studies need to be followed up by experiments using different species and routes of administration and by more extensive epidemiological studies, which in the case of benzene should not focus only on leukaemia but on all cancer morbidity and mortality. The authors make the additional point that the benzene study serves to validate the use of long-term animal studies to identify carcinogenic risk.

[It is unfortunate that these important studies are not as thoroughly reported as might be hoped; the lack of statistical analysis of the data is a serious omission, which makes it difficult to assess fully the significance of the results.]

Styrene causes SCE in mice ...

Conner, M. K., Alarie, Y. & Dombroske, R. L. (1980). Sister chromatid exchange in murine alveolar macrophages, bone marrow, and regenerating liver cells induced by styrene inhalation. *Toxic. appl. Pharmac.* **55,** 37.

Styrene, a widely used industrial chemical, can be metabolized in the body to form styrene oxide, which has been shown to be mutagenic (*Cited in F.C.T.* 1980, **18,** 434). Contradictory evidence has, however, been presented concerning the mutagenicity of styrene itself in several submammalian systems (*ibid* 1980, **18,** 434; Busk, *Mutation Res.* 1979, **67,** 201). The investigation by Conner *et al.* (cited above) was carried out to determine the relationship between short-term inhalation exposure to styrene and sister-chromatid-exchange (SCE) induction in murine alveolar macrophages, bone-marrow cells and regenerating liver cells.

Partially hepatectomized and non-hepatectomized mice are exposed to styrene concentrations of 104, 387, 591 or 922 ppm for 6 hr daily on 4 consecutive days. The highest concentration was also used for 6-hr exposures on 1 or 2 days. Immediately after the last exposure, bromodeoxyuridine (BrdU) incorporation was initiated so that SCE frequencies could be determined. Since BrdU incorporation occurs only in actively dividing cells, SCE analysis in liver cells was restricted to hepatectomized mice.

Dose-dependent increases in SCE frequencies were observed at concentrations of 387 ppm and above in all three types of cells in hepatectomized mice and in both the alveolar macrophages and bone-marrow cells in non-hepatectomized mice. In addition, at the highest concentration, increases in frequency were also found to be dependent on length of exposure. Since there was no significant difference between the SCE frequencies in bone-marrow cells or alveolar macrophages of hepatectomized and the frequencies in non-hepatectomized animals exposed to similar concentrations, the observed effects were not thought to be due to the biotransformation of styrene to styrene oxide. Hepatectomized mice were, however, more sensitive than non-hepatectomized mice to the cellular toxicity of styrene. This was indicated by a decrease in the proportion of second division relative to first division metaphases which was observed in all three cell types of hepatectomized mice exposed for 4 days to 922 ppm and in the regenerating liver cells after 4 days of exposure of 591 ppm. This suggests a loss in the styrene detoxification mechanism in the hepatectomized mice.

... And styrene oxide is found carcinogenic in rats

Maltoni, C., Failla, G. & Kassapidis, G. (1979). First experimental demonstration of the carcinogenic effects of styrene oxide. Long-term bioassays on

Sprague–Dawley rats by oral administration. *Medna Lav.* **5,** 358.

Styrene oxide is widely used in the production of epoxy resins, for surface coating, for textile treatment and in the preparation of a wide range of chemicals such as agrochemicals, cosmetics and perfumes. As a result of this wide range of uses, many different populations may be exposed to the compound. Since styrene oxide is also produced in large quantities it is somewhat surprising that only relatively recently has a long-term oral study been undertaken to evaluate its carcinogenic potential. Earlier skin-application studies showed no increase in the incidence of cutaneous tumours (Van Duuren *et al. J. natn. Cancer Inst.* 1963, **31,** 41; Weil *et al. Am. ind. Hyg. Ass. J.* 1963, **24,** 305). However, styrene oxide has been found to be a potent mutagen in several test systems, with and without metabolic activation (*Cited in F.C.T.* 1980, **18,** 434).

The study cited above involved the treatment of groups of 13-wk-old Sprague–Dawley rats with doses of either 50 or 250 mg styrene oxide/kg. The material was given as a solution in olive oil by stomach tube and dosing was carried out once daily, 4–5 days/wk for 52 wk. The animals were then kept untreated until spontaneous death, when autopsy and histological examination of a number of tissues were carried out. The experiment was completed at 156 wk.

The preliminary findings (dealing with results up to wk 135 of the study) indicated that the target organ was the stomach; papillomas, carcinomas *in situ* and invasive carcinomas of the forestomach were observed in animals from both dose groups. Precursor lesions were often seen in the forestomachs of animals with or without tumours and secondary carcinomas were often observed in the liver. None of the control animals (treated with olive oil alone) developed forestomach epithelial tumours and the authors state that these types of tumours are very rare in the colony of rats used in their laboratory. They therefore consider that the results indicate that styrene oxide is a very potent, direct acting carcinogen. It is suggested that inhalation studies should be undertaken using a wider range of doses than were used in the present study in order to allow a more thorough evaluation of the level of risk involved in the use of styrene oxide. Epidemiological studies should also be carried out.

[An NCI press release of 21 September 1979 announced that styrene oxide was being tested in a carcinogenicity bioassay. The results of this, and the final results of the study cited above, must be awaited before an assessment of the carcinogenicity of styrene oxide can be made.]

Triethyl tin: reversible toxicity in rats

Squibb, R. E., Carmichael, N. G. & Tilson, H. A. (1980). Behavioral and neuromorphological effects of triethyl tin bromide in adult rats. *Toxic appl. Pharmac* **55,** 188.

The widespread oedema of the white matter of the nervous system induced by dosing with triethyl tin (TET) has previously been characterized in both adult and growing animals and although the mechanism by which this occurs has not been clarified, binding of TET to myelin had been discounted as an important factor (*Cited in F.C.T.* 1971, **9,** 892; ibid 1977, **15,** 258; ibid 1977, **15,** 363; Graham & Gonatas, *Lab. Invest.* 1973, **29,** 628; Watanabe, *Neurotoxicology* 1977, **1,** 317). Work with mice has suggested that the neurophysiological changes following TET treatment are not primarily due to the gross ultrastructural changes observed (Gerren *et al. Pharmac. Biochem. Behav.* 1976, **5,** 299). The aim of the study cited above was to identify and quantify systematically the functional changes in TET bromide-dosed animals during periods of treatment and recovery, and to correlate these with the sequence of neuromorphological changes that occurred.

In order to familiarize them with the procedure of gastric intubation, 40 male rats were given twice-weekly doses of 5 ml 15% ethanol/kg body weight for a total of three doses. Ten of these rats (control group) received three further identical treatments whilst the three test groups (ten animals in each) were given three subsequent doses of 1, 2 or 3 mg TET (in 15% ethanol)/kg. Body weights, 24-hr food and water consumption, limb-grip strength, and startle responsiveness to acoustic and air-puff stimuli were recorded before TET dosing began, 24 hr after the second and third doses and 4 wk after cessation of dosing. All of the survivors at the highest dose level and four rats from each of the other groups were killed after the third dose of TET for examination of the brain and sciatic nerve. Additional brain sections were taken from the remaining animals after the 4-wk recovery phase.

The first significant change occurred in the startle reflexes at the end of the first week of TET treatment, after the administration of two doses of TET and a further decrease in response occurred after the third dose. The response to sound stimuli returned to normal within the recovery period but the deficient response to the air-puff stimulus in the 2-mg/kg dose group persisted. Reduced food and water consumption observed in this dose group after treatments 2 and 3 continued into the first week of the recovery phase but were not apparent in the final week of recovery. The body weights of rats given 2 mg TET/kg were significantly decreased after 1 wk of recovery, but although this effect persisted at wk 4 of recovery it was considerably reduced during the recovery phase. No differences in body weights were apparent at the 1-mg/kg dose level compared with the control group. Toxic signs including loss of body tone, weakness, chromodacryorrhea and insensitivity to pain were observed at the highest dose level. Four deaths had occurred in this group by the end of the dosing phase and the remaining six rats were then killed. Neuromorphological examination of these rats and of those from the other groups that were killed after dose 3 revealed intramyelinic oedema, of dose-dependent severity, affecting all the major white-matter tracts of the central nervous system. However, there was neither neuronal necrosis in the brain nor intramyelinic oedema, axonal degeneration, or Schwann cell proliferation in the sciatic nerve.

The characteristic signs of TET toxicity that appeared at the 2-mg/kg dose level, during the second week of treatment were reversed by the end of the

recovery period. Hindlimb grip strength was significantly reduced in the 1- and 2-mg/kg dose groups after the third dose of TET and in the latter group after 1 wk of recovery. Forelimb weakness occurred after 2 wk of dosing in the lowest dose group and after 1 wk of dosing in the 2-mg/kg dose group, persisting into the first week of recovery. However, full strength was restored by wk 4 of the recovery period.

Thus, by the end of the recovery period, all the major signs of TET toxicity, with the notable exception of the deficient startle response, had disappeared. Examination of brain-tissue sections of rats killed after 4 wk recovery revealed that two out of three samples from the 1-mg/kg dose group could not be distinguished from controls but that those from the 2-mg/kg dose group were still moderately oedematous.

Therefore previous findings that damage to the peripheral nervous system follows and/or occurs to a lesser degree than to the central nervous system are confirmed; in the present study the limb weakness appeared late and disappeared early, and the altered startle response occurred early and persisted longer. The authors conclude that dysfunction of the peripheral nervous system occurs later than, and is of shorter duration than, dysfunction of the central nervous system, but that both are capable of recovery.

A risk-assessment method for pesticide residues

Oller, W. L., Cairns, T., Bowman, M. C. & Fishbein, L. (1980). A toxicological risk assessment procedure: a proposal for a surveillance index for hazardous chemicals. *Archs envir. Contam. Toxicol.* **9**, 483.

In this paper a group of workers from the FDA's National Center for Toxicological Research, describe a procedure for assessing the degree of risk presented by residues of different pesticides in food or feed products. This technique can be applied in order to compile a priority list of chemicals for which residues should be monitored in certain food products, and to establish priorities for regulatory action.

The procedure involves the use of existing toxicological data to develop a numerical risk factor 'Surveillance Index' (SI) for the health hazard associated with a given chemical residue. The SI is the sum of the toxicity factor (TF), the environmental factor (EF) and the biosafety factor (BSF), the calculations of which are outlined below.

The TF is the product of the amount of the chemical applied to agricultural crops (including imported crops) each year and the toxicity (oral LD_{50} in rats) of the compound relative to dieldrin, a pesticide that is considered to be of well-established toxicity, and is designated moderately toxic, with a value of 1. Thus

$$TF = KT \times RTR$$

where KT is the amount of the compound released into the environment annually in ktons and RTR is the LD_{50} of dieldrin divided by the LD_{50} of the compound.

The EF is expressed as follows:

$$EF = CV \times t_{1/2E}$$

where CV is the sum of the crop values (defined below) and $t_{1/2E}$ is the effective half-life of the chemical calculated by the formula:

$$t_{1/2E} = (t_{1/2p} \times t_{1/2b})/(t_{1/2p} + t_{1/2b})$$

where $t_{1/2p}$ is the physical half-life and $t_{1/2b}$ is the biological half-life. The crop values are derived from values assigned to crops on the basis of the principle consumers, the time between harvest and consumption and the amount of preparation that is required before they are consumed. High values are given to crops that require little preparation such as fruit and certain vegetables, but low values are given to crops such as grain and animal feed. The physical half-life indicates the persistence of the chemical in the environment, and the biological half-life indicates its persistence in living systems.

The last term, the BSF, is calculated by the formula:

$$BSF = PB \times S \times PAR/NOEL$$

where PB is the propensity of the chemical to bioaccumulate, S is the specificity of the compound for reactive site(s) in man, PAR indicates the sector of the population at risk and NOEL is the presumed no observable effect level (ppm).

The PB provides a safety margin for compounds that can accumulate in the food chain or in man. If a compound bioaccumulates it is given a value of 2. If it does not it is given a value of 1. The specificity (S), is a subjective scale that defines the body burden that results from a particular compound. A compound that is not localized in the body is given a value of 2, one that has organ specificity is assigned a value of 5, and a DNA- or hormone-binding chemical is given an S value of 8. Thus the highest values are given to chemicals that attack the sites least able to tolerate the insult. To assign the PAR value the population is divided into three groups, the first comprises those with least capacity for metabolic detoxification, infants, children and the elderly, the second, teenagers and the third, adults, and these are given PAR values of 100, 80 and 50 respectively. The last element in the equation, the NOEL, is estimated by extrapolation from animal toxicity or epidemiological data. If data are insufficient to make a reasonable extrapolation to estimate the NOEL then the most hazardous situation should be assumed by using the lowest value from a class of structurally similar compounds.

Some examples of the results obtained using this risk-assessment method are given. On the basis of 1978 data, the SIs for 2,4,5-T, toxaphene, methyl parathion and carbaryl were calculated as 12,013, 1025, 310 and 160, respectively, and similar values were obtained using 1971 data. A modified SI can be calculated for compounds that are no longer in use, or that are naturally occurring or contaminant compounds (i.e. for which the amount released into the environment is unknown) using the formula:

$$\text{Modified SI} = (RTR \times EF) + BSF.$$

The modified SIs calculated for aflatoxins, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin, pentachlorophenol and polychlorinated biphenyls were 2077, 2000, 164 and 80, respectively.

The authors consider that the SI provides a feasible mechanism for selecting those chemicals that should be given priority in residue monitoring.

COSMETICS, PHARMACEUTICALS AND HOUSEHOLD PRODUCTS

Petrolatum—not an innocent bystander?

Penneys, N. S., Eaglstein, W. & Ziboh, V. (1980). Petrolatum: interference with the oxidation of arachidonic acid. *Br. J. Derm.* **103**, 257.

Petrolatum, a mixture of semi-solid hydrocarbons, is often used in skin tests as a physiologically inert matrix for the material under investigation. Whilst it has long been known that petrolatum can be beneficial in the treatment of burns and other wounds, the clinical improvement has generally been ascribed to physical effects such as removing debris and offering protection from irritants. However there have been some reports of petrolatum-induced adverse effects such as acanthosis (*Cited in F.C.T.* 1965, **3**, 138) and acne (*ibid* 1971, **9**, 763) which have suggested that petrolatum does have definite physiological activity. The report cited above adds to the evidence that ideas on petrolatum's biological inertness may need some revision.

The skin of anaesthetized pigs was scored to a depth of 0·3 mm with a dermatome. Petrolatum was applied daily for 7 days to one of the two wounded sites and to an area of undamaged skin. (Earlier studies had shown that re-epithelialization of both treated and untreated wounds occurred within 7 days.) At the end of the treatment period strips of skin were removed for assay. The petrolatum produced acanthosis when applied to the normal skin, but when applied to the wound it inhibited the hyperplastic response normally observed in an untreated wound. Prostaglandins and some hydroxy fatty acids are known to be pro-inflammatory and studies were carried out to determine whether petrolatum inhibited the formation of such substances in wounded skin. Microsomal preparations from normal untreated, and normal petrolatum-treated skin, and from petrolatum-treated and untreated wounds were tested for their ability to oxidize arachidonic acid as determined by oxygen uptake. Oxygen consumption by microsomes from normal skin were comparable, whether or not the skin had been treated with petrolatum. However, application of petrolatum to the wound significantly lowered the oxygen requirement of the resulting microsomes compared with those from the untreated wound. In experiments aimed at further defining this oxidizing activity the capability of the microsomes to convert [^{14}C]arachidonic acid into [^{14}C]-labelled prostaglandin and hydroxy fatty acids was monitored. There was no difference between microsomes from the petrolatum-treated and control wounds in their ability to convert arachidonic acid to prostaglandin, but microsomes from the treated wounds synthesized less hydroxy fatty acid than did those from the control wound. These data indicated that the decreased oxygenation was probably a reflection of a decreased microsomal lipoxygenase activity.

Experiments *in vitro* using microsomal preparations from foetal calf skin indicated a dose-related inhibition of prostaglandin synthetase by petrolatum. Because of the absence of such decreased activity in preparations of skin treated with petrolatum *in vivo* the investigators concluded that the inhibitory factor was not absorbed by the wounded skin to a significant extent. They suggest that the alteration of the cutaneous inflammatory response in damaged skin might instead be due to the local suppression of synthesis of hydroxy fatty acids, some of which are known to be pro-inflammatory.

No EDTA sensitization in the guinea-pig

Henck, J. W., Lockwood, D. D. & Olson, K. J. (1980). Skin sensitization potential of trisodium ethylenediaminetetraacetate. *Drug Chem. Toxicol.* **3**, 99.

Ethylenediaminetetraacetic acid (EDTA) and its salts are widely used as antioxidants and preservatives in many fields. In view of its use in cosmetics and pharmaceuticals, the finding that three out of 50 human volunteers (6%) tested in a skin sensitization study gave positive responses to the calcium salt (*Cited in F.C.T.* 1972, **10**, 699; Raymond & Gross, *Archs Derm.* 1969, **100**, 436) was disconcerting. In addition, two of these subjects were found to be cross-sensitized to ethylenediamine (ED). However, it was considered that these results did not necessarily reflect the likelihood of reactions within the general population and should be taken to indicate a weak skin-sensitizing potential only. This potential has now been re-evaluated in the guinea-pig.

Groups of ten male guinea-pigs were subjected to repeated insult patch testing (modified Maguire method) using 10% solutions of EDTA (trisodium salt), ED or the diglycidyl ether of 2,2-di-(*p,p'*-hydroxyphenyl)propane, in a solution of dipropylene glycol methyl ether and polyoxyethylene (20) sorbitan monooleate (9:1, v/v). Aliquots (0·1 ml) were applied topically to the clipped and depilated backs of the animals four times in 10 days. The third application was accompanied by an intradermal injection of 0·2 ml Freund's adjuvant, injected close to the test site. Two weeks afterwards 0·1 ml of the material tested originally was applied to one clipped flank of the guinea-pigs and solvent was applied to the other, except in the animals originally treated with ED, which were treated with EDTA (trisodium salt) instead of solvent. The treatment sites were examined 24 and 48 hr after these challenges.

The positive control, 2,2-di-(*p,p'*-hydroxyphenyl) propane (a known sensitizer in guinea-pigs and humans) produced slight to marked erythema and slight to moderate oedema in nine our of ten animals. All of those exposed to ED were sensitized but none of those treated with EDTA showed a positive response. No animal treated with ED and subsequently challenged with EDTA (trisodium salt) showed any evidence of cross-sensitization.

Therefore, whilst the existing data on the potency of ED as a skin sensitizer (*Cited in F.C.T.* 1976, **14**, 217) were confirmed, the suspicion concerning EDTA may prove to be unfounded. The authors conclude that the presence of very small amounts of the sodium salt of EDTA in skin preparations is unlikely to represent an appreciable risk to humans.

CARCINOGENICITY AND MUTAGENICITY

Gut feeling on the Ames test

Tamura, G., Gold, C., Ferro-Luzzi, A. & Ames, B. N. (1980). Fecalase: A model for activation of dietary glycosides to mutagens by intestinal flora. *Proc. natn. Acad. Sci. U.S.A.* **77**, 4961.

Talk of the Ames test conjures up images of a litmus-like paper which turns blue in contact with a carcinogen. Unfortunately things are not quite that simple and like many other bioassays, the quantitative estimation of histidine-revertants in selected strains of *Salmonella typhimurium* is markedly affected by slight changes in protocol. One of the most sensitive experimental parameters is the activating system added to the incubation mix as an approximation of mammalian metabolism. Although a liver microsomal fraction (S-9) obtained from PCB-induced rats is commonly used for this purpose, other systems are being developed. A recent paper from Bruce Ames' own group describes work on a new complementary activation system having the metabolizing power of the gut flora. This, they hope, will be useful in the mutagenicity testing of the glycosides found in edible plants which are hydrolysed by the bacteria in the human intestine but are not particularly susceptible to cleavage by the liver.

Advantage was taken of the fact that bacterial cells from the gut make up almost half of the weight of human faeces. Cellular debris was removed from the homogenized faeces by centrifugation and histidine and dithiothreitol, compounds that would interfere with the mutagenicity assay, were removed by gel chromatography. The resultant extract (fecalase) was shown to possess a wide range of glycosidase activity when tested with seven different glucosides. It was superior in this respect to hesperidinase, an extract of *Aspergillus niger*, and cecalase, a cell-free extract of rat caecal contents, two glycosidase fractions that have been used by other groups. In the mutagenicity studies, fecalase was added to standard strains of *Salmonella typhimurium*. None of the plant glycosides gave any evidence of mutagenic activity in Salmonella in the absence of metabolic activation. Although in the presence of fecalase, the only *C*-glucosides in the present study, aloin and carminic acid, were still inactive, the other glycosides tested, namely rutin, quercitrin, kaempferal, 8-hydroxyquinoline-β-D-glucoside, 8-hydroxyquinoline glucuronide, franguloside, robinin, neocycasin A and cycasin all proved to be mutagenic. The cycasin and quercitrin results correlated well with present knowledge on carcinogenicity, cycasin being a carcinogen in normal rats but not in germ-free rats which would lack the gut micro-organisms needed to cleave the sugar from the aglycone (Lacquer *et al. J. natn. Cancer Inst.* 1967, **39**, 355) whilst quercetin, the aglycone of quercitrin, has recently been shown to be a carcinogen in the rat (Pamukcu *et al. Proc. Am. Ass. Cancer Res.* 1980, **21**, 74).

A number of beverages thought to contain glycosides were evaluated in *S. typhimurium* (strain TA98). Black tea, a herb tea, red wine (California Burgundy) and grape juice were each potently mutagenic in the presence of fecalase. In its absence the teas and the grape juice were completely inactive, whereas the mutagenicity of the red wine was reduced by about half. This residual direct activity was thought to be due to the presence of quercetin formed in the wine during fermentation. White wine (California Chablis) and coffee were not mutagenic under any of the experimental conditions, indicating an absence of mutagenic flavonols or their glycosides. In earlier work from the Ames' laboratory, tea and other types of red wine (Californian zinfandel and a different Burgundy) exhibited mutagenicity in Salmonella when glusulase (taken from the snail) was added to the incubation mix. Japanese workers have also shown green and black tea to be mutagenic to *S. typhimurium* strain TA100 when activated with hesperidinase from *Aspergillus niger* (Nagao *et al. Mutation Res.* 1979, **68**, 101).

[Whilst these findings may well give some initial uneasy moments to the sellers of tea and red wine, they should not be received with unrelieved gloom by industry in general. If the fact that the glycosides in a culturally acceptable product such as a cup of tea are potently mutagenic in Salmonella can be imprinted on the public mind, there may be a more thoughtful response the next time an established industrial chemical is found to be Ames positive.]

Nitrosamines hit at guinea-pig liver

Cardy, R. H. & Lijinsky, W. (1980). Comparison of the carcinogenic effects of five nitrosamines in guinea pigs. *Cancer Res.* **40**, 1879.

Guinea-pigs have not been widely used in carcinogenicity studies. It is known that they are resistant to certain chemical carcinogens such as 2-acetylaminofluorene because they lack the enzyme system necessary to activate the carcinogen (Kawajiri *et al. Biochem. biophys. Res. Commun.* 1978, **85**, 959). In the study cited above the effects in guinea-pigs of five *N*-nitrosamines that are known to be potent carcinogens in rats or hamsters were investigated.

The five nitrosamines used were *N*-nitroso-2,6-dimethylmorpholine, *N*,*N*-dinitroso-2,6-dimethylpiperazine, *N*-nitrosomethyldodecylamine, *N*-nitrosoheptamethyleneimine and *N*-nitrosomethyldiethylurea. They were administered to groups of 20 male guinea-pigs by gavage twice weekly at doses roughly equivalent to those known to induce a significant number of tumours in rats or hamsters. Doses of 80 mg *N*-nitroso-2,6-dimethylmorpholine/kg body weight/wk proved to be toxic to the guinea-pigs and treatment was discontinued after 12 wk; the study was repeated using doses of 32 mg/kg/wk for 35 wk. Angiosarcomas of the liver developed in six of the guinea-pigs given the higher dose and in 19 of those given the lower dose of *N*-nitroso-2,6-dimethylmorpholine. Such tumours also occurred in 12 of the guinea-pigs given 200 mg *N*-nitrosomethyldodecylamine/kg/wk for 40 wk and in 18 of those treated with 96 mg *N*,*N*-dinitroso-2,6-dimethylpiperazine/kg/wk for 50 wk. Hepatocellular carcinomas were also observed in six guinea-pigs in the latter treatment group. Frequently, other hepatic lesions such as bile-duct carcinoma and/or cystic biliary fibroadenosis also occurred in guinea-pigs treated with one of these

three nitrosamines. However, no hepatic lesions were observed in animals treated with *N*-nitrosomethyldiethylurea (40 mg/kg/wk for 32 wk) and neither hepatic nor non-hepatic lesions were observed in guinea-pigs treated with *N*-nitrosoheptamethyleneimine (40 mg/kg/wk for 90 wk). Of the types of non-hepatic tumours that developed in the other four groups, two were thought to be treament related: four of the animals given *N*,*N*-dinitroso-2,6-dimethylpiperazine developed primary lung tumours and one brain tumour occurred after treatment with *N*-initrosomethyldiethylurea. The incidence of spontaneous tumours of these types is extremely low in guinea-pigs.

The liver was the primary target of the three nitrosamines for which there was a clearly demonstrated carcinogenic effect. Of the three *N*-nitrosodimethylmorpholine appeared to be the most potent, giving rise to tumours after a total dose of about 7 mmol/kg body weight compared with a total dose of about 30 mmol/kg for the other two. Although, because of differences in experimental conditions, it was not possible to make a precise comparison of the effectiveness of these nitrosamines in the guinea-pig with that in the rat or hamster, it appeared that the guinea-pig was less susceptible to all three than were the other two species. The authors point out that the target organ in the guinea-pig was the liver but that in hamsters and rats this organ is unaffected by any of the three nitrosamines. These differences might be the result of different metabolic pathways. However, many of the guinea-pigs died of liver toxicity and cancer early in the study and it is possible that at lower doses or with different patterns of exposure, non-hepatic tumours might have occurred later.

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LETTERS TO THE EDITOR

SCE IN MONKEYS FED IRRADIATED WHEAT

Sir,—Numerous short-term test systems have been used to evaluate the possible cytogenetic and mutagenic effects of irradiated foods, but the results have been inconsistent, ambiguous and difficult to reproduce (Barna, *Acta aliment.* 1979, **8,** 205). The International Project in the Field of Food Irradiation (Tech. Rep. no. IFIP-R53, 1979) has suggested the use of a battery of test systems for mutagenicity. The sister-chromatid exchange (SCE) assay offers a simple and accurate analysis of subtle alterations in chromosomes (Wolff, *A. Rev. Genet.* 1977, **11,** 183), but so far there have been no reports on the behaviour of this cytogenetic parameter in animals given irradiated foods. Therefore, we have studied SCE frequency in monkeys fed a diet containing freshly irradiated wheat.

When 16–18 months old, ten monkeys, *Macaca mulatta*, were divided equally into two groups fed diet containing unirradiated or freshly irradiated wheat. Fresh batches of wheat were irradiated at 20-day intervals, with a radiation dose of 75,000 rads from a cobalt-60 source, as described earlier (Vijayalaxmi & Sadasivan, *Int. J. Radiat. Biol.* 1975, **27,** 135), the dose being monitored by ferrous sulphate dosimetry. Both diets contained 70% wheat, 20% starch, 5% oil, 4% salt mixture and 1% vitamin mixture. After these diets had been fed for 12 months, blood samples were collected by venepuncture. Lymphocytes in whole blood were cultured in the dark at 37°C for 72 hr in RPMI-1640 medium, supplemented with 20% foetal calf serum, 100 units penicillin/ml, 100 μg streptomycin/ml, 2 mM-L-glutamine and 1% phytohaemagglutinin (Wellcome Research Laboratories, Beckenham, England). Bromodeoxyuridine at a final concentration of 10 μM was added to all the tubes at the beginning of culture. Colchicine (0·5 μg/ml) was present during the last 2 hr of incubation and chromosome preparations were made as described previously (Murthy & Prema, *Mutation Res.* 1979, **68,** 149). Air-dried slides, aged for 1 day, were stained for SCE using the Hoechst–Giemsa method (Perry & Wolff, *Nature, Lond.* 1974, **251,** 156). SCEs were scored in 25 M2 cells on coded slides by a single observer.

Monkeys fed the diet containing freshly irradiated wheat showed a mean SCE frequency (± SEM) of 5·89 ± 0·54, which did not differ to a statistically significant degree (Student's *t* test) from the mean of 5·67 ± 0·41 for monkeys fed unirradiated wheat.

While these results show that there is no increase in the frequency of SCE in monkeys fed irradiated wheat, it should be stressed that this observation does not settle the issue regarding the presence of polyploid cells in animals fed irradiated wheat (Vijayalaxmi, *Toxicology* 1978, **9,** 181). It is well known that not all mutagens and carcinogens induce SCE (Latt, *Genetics* 1979, **s92,** 83; Wolff, *A. Rev. Genet.* 1977, **11,** 183), and since SCE is a more sensitive parameter than the analysis of structural chromosomal aberration (Perry & Evans, *Nature, Lond.* 1975, **258,** 121), its comparison with polyploidy, a numerical aberration, is unwarranted. However, neither the significance of SCE nor of polyploidy is known to us.

Recently Phillips *et al.* (*Fd Cosmet. Toxicol.* 1980, **18,** 471) have reported that cultured Chinese hamster ovary cells treated with extracts of irradiated foods did not show altered SCE rates.

The author is grateful to Dr P. G. Tulpule, Director, National Institute of Nutrition, for guidance.

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USE OF S-9 MIX IN MUTAGENICITY TESTS OF URINE CONCENTRATES

Sir,—Dr Crebelli and his colleagues (*Fd Cosmet. Toxicol.* 1981, **19**, 79) are to be commended for attempting to unravel some of the complexities associated with the mutagenicity or non-mutagenicity of various *p*-phenylenediamine (*p*-PD) samples and oxidation mixtures derived from them. Considerable scope undoubtedly exists for the application and development of short-term methods to explore various aspects of toxicity in depth without resorting to laborious and time-consuming long-term *in vivo* techniques. If research on these lines is to become a major component of safety evaluation, however, it must be unequivocally rational as well as rigorous in scientific discipline.

The rationality of the work reported by Crebelli *et al.* (*loc. cit.*) is called into question when Table 3 in the paper is examined. The urine concentrate from a rat treated topically with 300 mg *p*-PD/resorcinol conjugates was clearly not mutagenic under the conditions of experiment. The authors proceeded to study the effect of a rat liver S-9 mix on the same urine concentrate, which was then found to induce mutations in the TA98 strain of *Salmonella typhimurium*. Since an environmental chemical will already have undergone any likely transformation by liver enzymes under *in vivo* conditions before it reaches the urine, it is difficult to see any reason for arranging a further encounter with liver enzymes. Perhaps the finding might be held to bear some relationship to the 'ultimate' toxic potential; nevertheless these findings totally ignore pharmacokinetic considerations appertaining to the material under investigation and therefore necessarily defy any attempt at interpretation.

It could perhaps be argued that metabolites having mutagenic potential, though not normally reaching detectable levels, might be produced in particular circumstances whereby liver-enzyme activity is enhanced. If this is a cause for concern, the rational approach would surely be to pre-treat the animals with a recognized enzyme-inducing agent such as phenobarbitone. At least, this would ensure that biotransformation of the test material bore some reasonable relationship to events that could conceivably occur in real life.

N. J. Van Abbé,
Beecham Products Research Department, Randalls Road, Leatherhead, Surrey, KT22 7RX, England

Sir,—In our work (*Fd Cosmet. Toxicol.* 1981, **19**, 79), the principal aim of the urine mutagenicity assay was neither the quantitative assessment of risk nor a study of pharmacokinetics, but the demonstration of the absorption of a mutagenic chemical into the body by the detection of its stable or conjugated metabolites and/or of that fraction of unmodified compound that had not reacted with nucleophilic targets.

It is well known that the major limitation of the urine assay is that the most reactive metabolites, which are usually the most interesting from the toxicological point of view, are not usually found in the urine. One typical example of this phenomenon is described by Durston & Ames (*Proc. natn. Acad. Sci. U.S.A.* 1974, **71**, 737): when rats are fed with 2-acetylaminofluorene, its hydroxylated metabolites appear in the urine (mainly as glucuronides), but even after treatment of the urine with β-glucuronidase, further metabolism, i.e. the presence of S-9 mix, is required if high mutagenic activity is to be observed.

Accordingly, when we attempted to demonstrate the percutaneous absorption of the compound under test, we considered it rational to use an *in vitro* activation system for the detection of the unmetabolized fraction of a compound that we had shown required metabolic activation to exert its mutagenic activity.

Mr Van Abbé will agree that omitting the use of S-9 mix in the urine assay would have provided a wrong or inconclusive answer to our question regarding the percutaneous absorption of the reaction product between *p*-phenylendiamine and resorcinol.

Furthermore we can conclude from the previous considerations that the use of animals pretreated with enzyme inducers would be particularly unfavourable for our aim, as the increased hepatic metabolism could suppress the urinary recovery of the chemical studied, by totally or almost totally transforming it into reactive species which would thus remain completely on the macromolecular targets.

R. Crebelli and A. Carere
Istituto Superiore di Santà, Rome
and
R. Zito
Istituto Regina Elena, Rome, Italy

Fd Cosmet. Toxicol. Vol. 19, p. 525
Pergamon Press Ltd 1981. Printed in Great Britain

ANNOUNCEMENT

EXPERIMENTAL PATHOLOGY ESSAY PRIZE

The Experimental Pathology Club is offering a prize of £100 for an essay or paper (published or unpublished) embodying original research on some aspect of pathogenesis by a student or graduate under 30 years of age. The closing data for entries is 31 January 1982 and further particulars may be obtained from the Honorary Secretary of the Club, Dr R. A. Little, MRC Trauma Unit, Stopford Building, Oxford Road, Manchester M13 9PT.

Fd. Cosmet. Toxicol. Vol. 19, p. 526
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MEETING ANNOUNCEMENTS

MICROPOLLUTANTS IN THE ENVIRONMENT

A conference on micropollutants in the environment is to be held on 22–25 November 1981 in the Brussels Exhibition Centre. The conference will deal with the occurrence and effects of micropollutants in ground and waste water, their elimination, and the methodology of research concerning micropollutants. Requests for further information should be addressed to: Brussels International Conference Centre, Parc des Expositions, B-1020 Brussels, Belgium (Telex 23643).

SYMPOSIUM ON DRUG METABOLISM

The UK Drug Metabolism Group is organizing a residential meeting on aspects of drug metabolism at Stowe School, near Buckingham, on 4–7 April 1982. The main sessions will cover pathways of metabolism and their control, chemical aspects of drug metabolism, enzyme induction and recognition and significance of multiple forms of drug metabolizing enzymes. There will also be poster sessions. The conference fee inclusive of all meals and dormitory style accommodation will be £70. This fee will be reduced to £60 for a limited number of registered research students and research fellows. For further details contact Dr J. Caldwell, Department of Biochemical and Experimental Pharmacology, St Mary's Hospital Medical School, Paddington, London W2 1PG.

Fd. Cosmet. Toxicol. Vol. 19, p. 527
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FORTHCOMING PAPERS

The next issue of *Food and Cosmetics Toxicology* will be a Special Issue in honour of Dr L. Golberg and to mark the twentieth anniversary of the founding of BIBRA. It is hoped to publish the following papers in the Special Issue:

Bioassay for evaluating the potency of airborne sensory irritants and predicting acceptable levels of exposure in man. By Y. Alarie.

The role of zinc in nitrilotriacetate (NTA)-associated renal tubular cell toxicity. By R. L. Anderson.

The delayed long-term effects of chemicals following neonatal exposure in laboratory animals. By T. Balazs.

Action of *N*,*N*-diethylacetamide on hepatic microsomal drug-metabolizing enzymes. By F. E. Beyhl and E. Lindner.

Review of the hepatic response to hypolipidaemic drugs in rodents and assessment of its toxicological significance to man. By A. J. Cohen and P. Grasso.

Interspecies response to carcinogens and oestrogens. By V. A. Drill.

Sulphite toxicity: A critical review of *in vitro* and *in vivo* data. By A. F. Gunnison.

The impact of air-lead on blood-lead in man—a critique of the recent literature. By P. B. Hammond.

Rethinking the environmental causation of human cancer. By J. Higginson.

Structure-mutagenicity analysis with the CHO/HGPRT system. By A. W. Hsie.

Safrole: Its metabolism, carcinogenicity and interactions with cytochrome *P*-450. By C. Ioannides, M. Delaforge and D. V. Parke.

Failure of chloroform to induce chromosome damage or sister-chromatid exchanges in cultured human lymphocytes and failure to induce reversion in *Escherichia coli*. By D. J. Kirkland, K. L. Smith and N. J. van Abbé.

Studies on the mechanism of carcinogenicity of diethylstilboestrol: Role of metabolic activation. By M. Metzler.

Risk assessment and regulatory decision making. By I. C. Munro and D. R. Krewski.

Genetic and nongenetic events in neoplasia. By W. T. Stott, R. H. Reitz, A. M. Schumann and P. G. Watanabe.

The decision-point approach for systematic carcinogen testing. By J. H. Weisburger and G. M. Williams.

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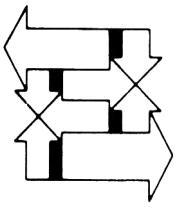

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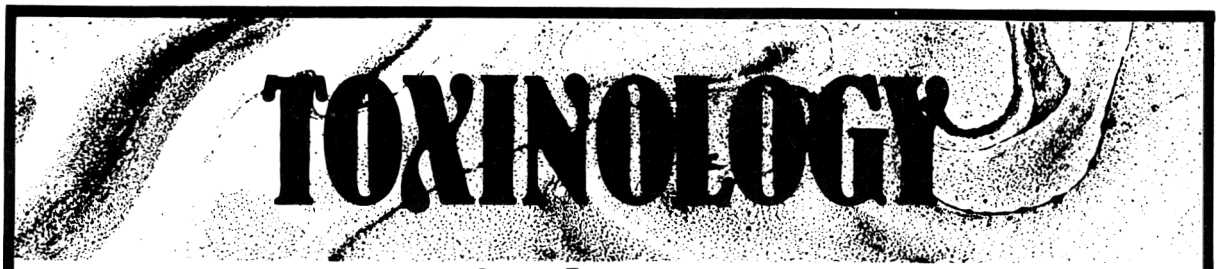

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