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Research Section

STUDIES ON THE METABOLISM OF SUCROSE ACETATE ISOBUTYRATE IN THE RAT AND IN MAN

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(*Received 25 February 1976*)

Abstract—*In vitro* and *in vivo* studies in the rat and in man were conducted on the metabolic fate of sucrose acetate isobutyrate (SAIB). *In vitro* studies with rat tissues showed that SAIB was substantially hydrolysed by non-specific esterases associated with the intestinal mucosa; little hydrolysis occurred in the stomach and liver. In the intact animal, orally administered [^{14}C]SAIB was found to be hydrolysed in the gastro-intestinal tract prior to absorption, and the subsequent fate of administered radioactivity was analogous to that of [^{14}C]sucrose. Human studies showed that, following a single oral dose or seven daily doses of 1 g SAIB, the urine contained no sucrose or partially hydrolysed SAIB. Microflora associated with the large bowel were found to exhibit a limited capacity to hydrolyse SAIB. The results show that the use of SAIB as a food additive is unlikely to constitute a toxicological problem with respect to its metabolic fate in man.

INTRODUCTION

The use of brominated vegetable oil as a suspending agent for flavouring oils in soft drinks was banned in the United Kingdom in 1970 (Statutory Instrument 1970, no. 1101). Sucrose acetate isobutyrate (SAIB) was found to be a technically suitable alternative. It is a colourless, tasteless, highly viscous mixture of sucrose esters containing approximately 2 mol acetate and 6 mol isobutyrate/mol sucrose. The major constituent of the mixture is 6,6′-diacetyl-2,3,4,1′3′4′-hexa-isobutyryl sucrose.

The results of studies using a number of animal species have indicated that the acute and chronic toxicity of SAIB is low. Oral and ip LD_{50} values greater than 25 g/kg in rats and mice have been reported by Krasavage, DiVincenzo, Astill, Roudabush & Terhaar (1973). Experiments in which rats were fed a diet containing up to 10% (w/w) SAIB have revealed no significant adverse effects. Feeding studies with beagle dogs have shown some effects on the liver, including increased absolute weight and a prolonged clearance time for indocyanin green, but all the effects were reversed when SAIB was withdrawn from the diet (Krasavage *et al.* 1973; Procter, Dussault & Chappel, 1973).

Studies on the disposition and metabolism of SAIB indicate that the utilization of SAIB in man is similar to that in the rat but significantly different from that in the dog (Reynolds, Astill, Terhaar & Fassett, 1974). We have, therefore, compared in more detail the metabolic fate of SAIB in the rat and in man and have paid particular attention to the role of the gastro-intestinal microflora so that a better assessment of the safety-in-use of the material may be made.

EXPERIMENTAL

Materials. SAIB was supplied by Bush Boake Allen Ltd., London. [U-^{14}C]Sucrose acetate isobutyrate ([^{14}C]SAIB), specific activity 1·007 mCi/g, was supplied by Eastman Kodak Co., New York, and was used undiluted. [U-^{14}C]Sucrose, specific activity 600 mCi/mmol, was purchased from the Radiochemical Centre, Amersham, Bucks., silica gel G from E. Merck AG, Darmstadt, and all bacteriological media from Oxoid Ltd., London.

Animals. Both sexes of Wistar albino rats, obtained from a specified-pathogen-free colony, were maintained on Spillers' Laboratory Small Animal Diet SG1 and water *ad lib.* and were housed at 20 ± 1°C.

Chromatography. Urine extracts were chromatographed on Whatman No. 1 paper and developed (Smith, 1960) with isopropanol–water (4:1, v/v). ^{14}C-labelled extracts of urine, faeces and tissue homogenates were chromatographed on a thin layer of silica gel G applied to plates from a suspension in 0·1 M-phosphate buffer (pH 5), the plates being developed with benzene–methanol (24:1, v/v), solvent A, or benzene–methanol (4:1, v/v), solvent B. [^{14}C]Sucrose was chromatographed on silica gel G using *n*-butanol–acetone–0·1 M-phosphate buffer, pH 5·0 (4:5:1, by vol.), solvent C (Lewin & Smith, 1969). Reducing sugars separated by paper chromatography were visualized by spraying with aniline–diphenylamine–phosphoric acid (Buchan & Savage, 1952). Sucrose and sucrose esters separated by thin-layer chromatography were visualized by spraying with resorcinol–phosphoric acid. ^{14}C-labelled compounds were analysed by scintillation counting of silica gel removed from sections of the plates.

Radioactivity measurements. Tissue homogenates were solubilized with NCS solubilizer (Amersham-Searle Corp., Ill., USA) and counted in a scintillation fluid of toluene–2-ethoxyethanol (1:1, v/v) containing PPO (0·3%) and POPOP (0·03%). Silica gel from thin-layer chromatographs was counted in a toluene scintillator containing 4% (w/v) Cab-O-Sil (Koch Light). Radioactivity was measured in a Nuclear Chicago

Mk 1 Liquid Scintillation Counter, and the efficiency was determined by the external channels-ratio method (Baillie 1959).

Hydrolysis studies. In all hydrolysis studies, control incubations of SAIB with autoclaved samples of the appropriate tissue were carried out under conditions identical to those with the non-autoclaved tissue.

Animal studies

Incubation with homogenates of gut contents. Animals were killed by a blow on the head, and the alimentary tract was ligated to separate the stomach, small intestine and caecum. The contents of the three regions were expressed into 1-oz McCartney bottles and dispersed in 4 vol. of medium, which contained 0·2 M-potassium phosphate buffer (pH 7·0) and 25% (v/v) Ringers solution. The resulting suspensions were centrifuged at 16 **g** for 1 min to remove large debris. An aliquot (5 ml) of each suspension was transferred to a screw-capped bottle containing 0·05 ml [^{14}C]SAIB in ethanol (10 mg/ml). The bottles were incubated at 37°C for 6 hr under a stream of oxygen-free nitrogen to maintain anaerobic conditions. At the end of the incubation the mixture was deproteinized with 0·5 vol. 5% w/v aqueous $ZnSO_4$ and 0·5 vol. saturated aqueous $Ba(OH)_2$ and centrifuged. The supernatant liquid was removed, and the precipitate was washed with ethanol. The combined supernatant liquid and washings were made up to 10 ml, and aliquots (0·5 ml) were analysed for SAIB and metabolites by thin-layer chromatography (TLC) in solvent systems A, B and C.

Incubation with homogenates of liver and intestinal mucosa. Animals were killed by a blow on the head, and the liver and small intestine were removed. Homogenates of the liver and the intestinal mucosa (10%, w/v) were prepared in Krebs–Ringer phosphate buffer (pH 7·4) containing glucose (0·1%, w/v). Aliquots (3 ml) of the suspension were incubated with [^{14}C]SAIB at either 25 or 250 μg/ml. At 0, 1, 2 and 4 hr, portions (0·5 ml) were removed and assayed for SAIB and metabolites by TLC.

Whole-animal metabolic studies. Female rats were given, by oral intubation, [^{14}C]SAIB (50 mg/kg) dissolved in corn oil (6 ml/kg). Following administration, the animals were housed individually in all-glass metabolism cages (Metabowl, Jencon Scientific Ltd., Hemel Hempstead) and allowed free access to food and water. Air was drawn through the system at a constant rate of 250 ml/min and exhaled $^{14}CO_2$ was trapped in ethanolamine–2-ethoxyethanol (1:4, v/v). Animals were killed at 6 or 24 hr, and the total radioactivity excreted in the faeces and urine and as $^{14}CO_2$ was determined. Additionally, the concentration of radioactivity in the stomach, small intestine, caecum, colon, liver and kidney was determined, and the distribution of [^{14}C]SAIB and its hydrolysis products was examined in the contents of the stomach, small intestine and caecum of the rats at 6 hr and in the urine and faeces at both time intervals by TLC. A single female rat was given [^{14}C]sucrose (20 mg/kg; 2·5 μCi/kg) by oral intubation, and the urine was collected over 24 hr. Aliquots of the urine (0·5 ml) were examined by TLC in solvent systems A and B.

To investigate the significance of the caecum as a site of absorption and hydrolysis of SAIB, [^{14}C]SAIB (20 mg/kg) dissolved in corn oil (0·1 ml) was introduced directly into the caecum of male rats. The rats were anaesthetized with ether and a small incision was made in the lower abdomen. Following ligation of the caecum from the small intestine, [^{14}C]SAIB was injected into the centre of the caecum, the incision was stitched up and the rat was placed in a metabolism cage. Rats were killed at 3 or 6 hr, and the extent of metabolism was investigated as described above.

Intestinal absorption studies. Absorption was studied using the technique described by Matthews, Craft, Geddes, Wise & Hyde (1968). Animals were anaesthetized with sodium pentobarbitone, and a 25-cm length of small intestine was isolated between ligatures. Three separate 5-cm sections were isolated within this length and 0·25 ml of an emulsion, made by sonication of [^{14}C]SAIB (0·4 mg) or [^{14}C]sucrose (0·15 mg) with tragacanth in saline, was introduced into each loop. After absorptive periods of up to 1 hr, the loops were excised and washed out with 0·9% (w/v) saline solution. Portions of the wall homogenate and combined contents and washings were assayed for radioactivity.

Human studies

Incubation with faecal homogenates. Human faeces were homogenized in 0·2 M-phosphate buffer (pH 7·0) containing 25% (v/v) Ringers solution to give a final concentration of 0·2 g faeces/ml. Aliquots (2 ml) of the suspensions were incubated under N_2 at 37°C with [^{14}C]SAIB solution to give an initial concentration of SAIB of 0·1 or 1 mg/ml. Portions (0·1 ml) were removed at intervals up to 16 hr and analysed for SAIB and metabolites by TLC.

Isolation of bacteria and preparation of suspensions. Bacteria were isolated from human faeces using selective media similar to those described by Drasar (1967). Cultures of *Escherichia coli* and *Streptococcus faecalis* were grown to mid-exponential phase in Nutrient Broth No. 2, centrifuged at 10,000 **g** for 20 min at 4°C and re-suspended in 0·2 M-phosphate buffer (pH 7·0) at a final cell density of 2×10^9 viable cells/ml. Suspensions of Lactobacillus strains in the same buffer were derived from mid-exponential phase cultures in MRS Broth. Strains of bacteroides and bifidobacteria were grown on Reinforced Clostridial Medium for 2–3 days in an atmosphere of H_2–CO_2 (9:1) and suspended as described above. The bacterial suspension (2 ml) was incubated with [^{14}C]SAIB (100 μg/ml) for 20 hr under a stream of N_2 at 37°C. Hydrolysis of SAIB was followed as described above.

In vivo studies. In all the experiments, the volunteers were given SAIB dissolved in butter. Urine was examined for sucrose by paper chromatography. Aliquots were examined for free sucrose directly and for total sucrose (free and esterified) after hydrolysis with 0·167 N-KOH. Aliquots (10 ml) were clarified with activated charcoal (Norit A, Hopkin & Williams), evaporated to dryness and redissolved in water (0·2 ml). Portions of the concentrated urine (40 μl) were chromatographed against known concentrations of sucrose and the intensity of the spots were compared after development with the aniline–diphenylamine–phosphoric acid spray reagent.

In the single-dose study, two male subjects were given SAIB (0·1 and 1 g), and 24-hr urine samples were collected for 5 consecutive days. Portions of the urine were examined for free and esterified sucrose. In the multiple-dose study, two male subjects were given 1 g SAIB for 7 days, and 24-hr urine samples were collected and examined for total sucrose.

To study faecal excretion, a single subject was given 0·1 g SAIB for 7 days and total faeces were collected during the subsequent 3 days. The 24-hr faecal samples were dried, and the lipid material was extracted twice with diethyl ether in a Soxhlet apparatus. The ether extract was evaporated, dissolved in ethanolic NaOH (0·4 M in 96% ethanol) and heated under reflux for 1 hr. The solution was evaporated to dryness, the residue was taken up in water (10 ml) and acidified with conc. HCl, the fat was extracted with petroleum ether, and the aqueous phase was boiled for 15 min. After neutralization, the aqueous solution was analysed for glucose by the method of Asatoor & King (1954).

The clearance of sucrose in the urine was studied in two subjects each given 100, 250 and 500 mg sucrose iv as a 10% (w/v) solution on different days. Total urine was collected at 3, 12 and 24 hr, the samples were clarified with activated charcoal, and glucose concentration was determined after acid hydrolysis.

RESULTS

Animal studies in vitro

The degradation of SAIB under anaerobic conditions by the contents of different regions of the intestinal tract of the rat is shown in Fig. 1. The results show that the activity associated with the proximal region of the small intestine was greatest, with approximately 75% hydrolysis of SAIB by 6 hr. The activity associated with the distal small intestine was similar, although the extent of hydrolysis to compounds with low chromatographic mobility was less. The activity of the caecal contents was substantially less than that of the small intestine, and the stomach contents showed no hydrolytic activity.

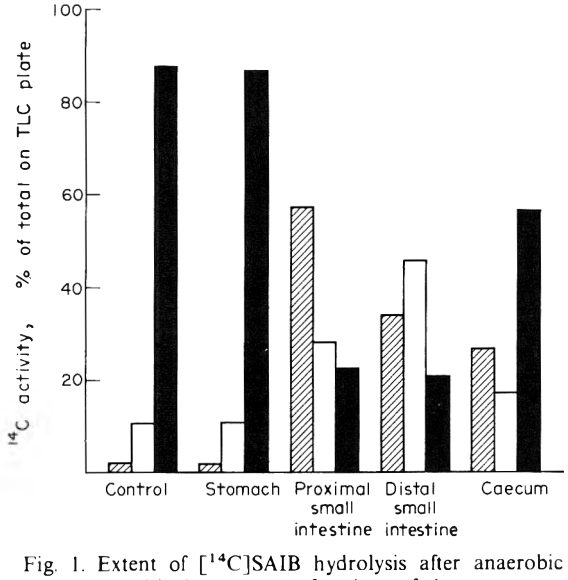

Fig. 1. Extent of [^{14}C]SAIB hydrolysis after anaerobic incubation with the contents of regions of the rat gastrointestinal tract. The bars represent ^{14}C activity chromatographing with an $R_F < 0{\cdot}10$ ▨, 0·10–0·95 □ and >0·95 ■. Decreasing R_F value below that of SAIB (>0·95) represents increasing hydrolysis.

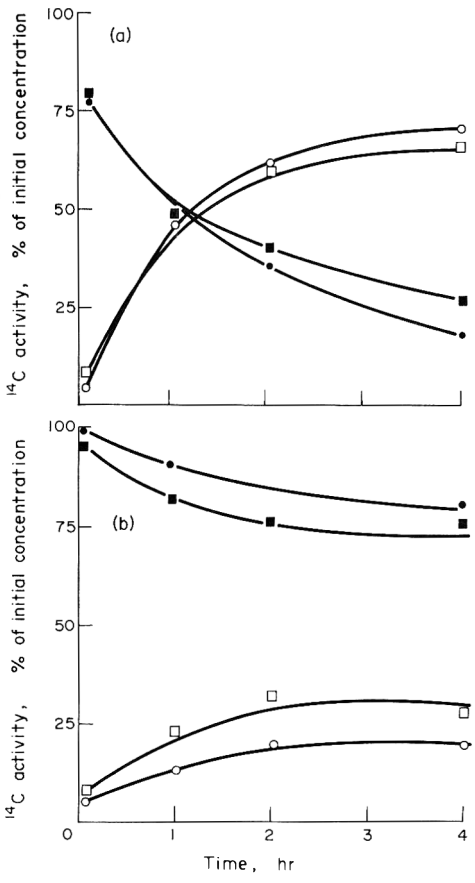

Fig. 2. Aerobic hydrolysis of [^{14}C]SAIB (■,●) by homogenates of rat intestinal mucosa (a) and liver (b) and associated increase in concentration of low-molecular-weight esters (□,○) in incubates containing SAIB at an initial concentration of 25 (■,□) or 250 (●,○) μg/ml. Each point represents the mean for two experiments.

The hydrolysis of SAIB by homogenates of intestinal mucosa under aerobic conditions is shown in Fig. 2a. The rate and extent of hydrolysis was similar when the SAIB was present at an initial concentration of either 25 or 250 μg/ml. In contrast, the rate and extent of hydrolysis of SAIB by homogenates of liver, shown in Fig. 2b, was less than that of intestinal mucosa, and both parameters were dependent on the initial concentration. At 25 μg/ml there was less than 30% hydrolysis of SAIB while at 250 μg/ml, there was less than 20% hydrolysis.

Animal studies in vivo

The distribution of SAIB and its metabolites at 6 and 24 hr after the oral administration of [^{14}C]SAIB is shown in Table 1. More than 90% of the radioactivity remained in the intestinal tract at 6 hr, with approximately 60% in the small intestine. Considerable hydrolysis had occurred, however, with less than 30% of the radioactivity recovered from the small intestine and caecum chromatographing with SAIB. The extent of hydrolysis to sucrose and other metabolites of low

Table 1. *Distribution of radioactivity following oral administration of [^{14}C]SAIB to male rats*

Sample	6 hr after intubation			24 hr after intubation		
	Total ^{14}C activity recovered (%)	^{14}C activity (% of recovered dose) chromatographing with		Total ^{14}C activity recovered (%)	^{14}C activity (% of recovered dose) chromatographing with	
		SAIB	Sucrose		SAIB	Sucrose
Stomach contents	6·9	91·9	0·5	0·8	ND	ND
Small intestine contents	61·7	29·5	4·3	0·9	ND	ND
Caecum contents	15·1	24·5	16·7	2·9	ND	ND
Colon contents	1·6	ND	ND	1·0	ND	ND
Faeces*	—	—	—	33·3	24·2	11·2
Urine	4·9	3·8	58·9	12·0	0·7	69·4
CO_2	2·4	—	—	45·1	—	—
Gut wall	5·9	ND	ND	1·9	ND	ND
Liver and kidney	1·4	ND	ND	2·1	ND	ND

ND = Not determined

*No faeces at 6 hr.
Values are means for two rats.

chromatographic mobility was greatest in the caecum. Urinary excretion of radioactivity accounted for only 4·9% of the dose and metabolism to $^{14}CO_2$, representing complete hydrolysis to glucose and fructose, for only 2·4% of the dose. In contrast to these findings, 90% of the radioactivity had been excreted by 24 hr and less than 8% remained in the gastro-intestinal tract. Metabolism to $^{14}CO_2$ accounted for 50% of the excreted radioactivity. Radioactivity in the faecal material, of which 24% chromatographed with SAIB, accounted for 33% of the dose. The chromatographic distribution of radioactivity in the urine of rats treated with [^{14}C]SAIB was similar to that obtained from the urine of animals given an equivalent dose of [^{14}C]sucrose.

Table 2 shows the distribution of radioactivity and extent of metabolism of [^{14}C]SAIB 3 and 6 hr after injection of the compound into the ligated caecum

Table 2. *Distribution of radioactivity following intracaecal injection of [^{14}C]SAIB in male rats*

Sample	3 hr after injection			6 hr after injection		
	Total ^{14}C activity recovered (%)	^{14}C activity (% of recovered dose) chromatographing with		Total ^{14}C activity recovered (%)	^{14}C activity (% of recovered dose) chromatographing with	
		SAIB	Sucrose		SAIB	Sucrose
Stomach contents	1·0	ND	ND	0·2	ND	ND
Intestine contents	0·8	ND	ND	3·6	ND	ND
Caecum contents	84·1	87·6	0·9	74·2	85·6	1·9
Colon contents	0·6	ND	ND	12·9	79·2	2·8
Faeces*						
(Benzene extract)	—	—	—	0·9	66·1	5·5
(Aqueous extract)	—	—	—	0·4	ND	ND
Urine	0·03	ND	ND	1·1	ND	ND
CO_2	0·2	—	—	3·2	—	—
Gut wall	13·1	ND	ND	2·9	ND	ND
Liver & kidney	0·2	ND	ND	2·7	ND	ND

ND = Not determined

*No faeces at 3 hr.
Values are means for two rats.

Table 3. *Absorption of [^{14}C]sucrose and [^{14}C]SAIB from isolated loops of rat small intestine*

Compound	^{14}C activity remaining in loop after		
	0 min	30 min	60 min
[^{14}C]Sucrose (0·4 mg)	99·4 ± 1·6 (3)	28·9 ± 7·8 (3)	ND
[^{14}C]SAIB (0·15 mg)	102·0 ± 7·4 (3)	93·2 ± 1·1 (2)	87·2 ± 0·5 (2)

ND = Not determined
Values are means ± SD for the number of loops in parenthesis.

Table 4. *Anaerobic hydrolysis of [^{14}C]SAIB by human faecal homogenates*

Dose (mg/ml)	System	^{14}C activity (%) recovered as SAIB	Metabolites with R_F < 0·10*	Sucrose
0·1	Control	84·2, 86·8	0·9, 1·0	0·1, 0·4
	Faeces	50·1, 51·0	34·4, 34·8	1·9, 1·6
1	Control	81·0, 77·1	1·5, 4·4	0·0, 0·1
	Faeces	29·5, 28·9	57·7, 62·4	4·8, 5·1

*TLC System A (see Experimental).
Each value represents the recovery from a single incubation.

Table 5. *Hydrolysis of [^{14}C]SAIB by pure cultures of bacteria*

Organism	No of strains	SAIB remaining (%)	Partially hydrolysed metabolite formed (%)
Control	—	93·8	1·0
Escherichia coli	6	69·9–92·3	1·4–6·0
Streptococci	5	76·2–91·5	0·6–1·9
Lactobacilli	5	83·3–89·6	1·6–4·0
Bacteroides	5	71·8–94·0	2·1–6·2
Bifidobacteria	5	69·4–91·0	2·3–4·7

Figures for bacterial cultures represent the range of values obtained.

of male rats. At 6 hr, approximately 5% of the radioactivity had been excreted, predominantly as $^{14}CO_2$, with the caecum retaining 74%. The extent of hydrolysis in the caecum at this time was less than that found after oral administration (Table 1).

The disappearance of radioactivity from loops of small intestine after injection of [^{14}C]sucrose and [^{14}C]SAIB is shown in Table 3. Sucrose was cleared rapidly from the intestine, with less than 30% of the radioactivity remaining at 30 min. In contrast, the rate of removal of SAIB was very slow, 87% remaining after 1 hr.

Human studies in vitro

Table 4 and Fig. 3 show the hydrolysis of [^{14}C]SAIB by faecal homogenates under anaerobic conditions. In these experiments, unhydrolysed [^{14}C]SAIB migrates with an R_F value of >0·95 in TLC system A, and sucrose and the majority of metabolites migrate with an R_F value of <0·10. The material chromatographing with an R_F value of <0·10 was eluted from the silica gel and re-run in solvent system C to resolve sucrose and partially esterified sucrose esters.

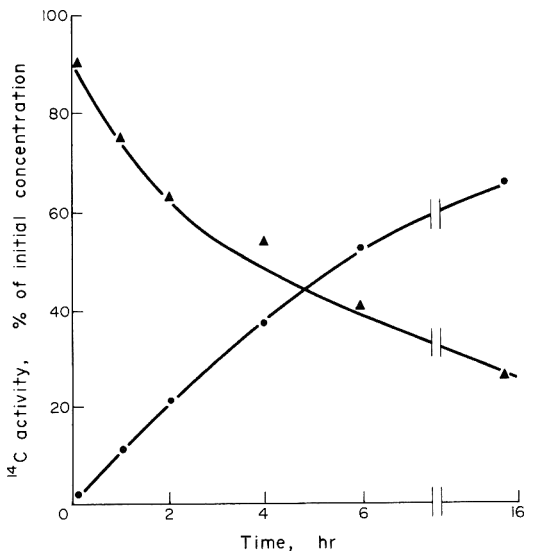

Fig. 3. Anaerobic hydrolysis of [^{14}C]SAIB (▲) by human faecal homogenates (0.1 mg SAIB/ml) and associated increase in concentration of sucrose and low-molecular-weight esters (●).

Incubation of [^{14}C]SAIB with human faecal homogenates (1 mg SAIB/ml) resulted in 40% hydrolysis by 16 hr, less than 2% of the [^{14}C]SAIB being hydrolysed completely to sucrose. With [^{14}C]SAIB present initially at a concentration of 0·1 mg/ml, the extent of hydrolysis was greater, 60% of the [^{14}C]SAIB being hydrolysed and 5% completely de-esterified.

The hydrolysis of SAIB by suspensions of human-gut bacteria isolated from faeces (Table 5) was considerably less than that by suspensions of faecal homogenate under similar conditions. Only two strains each of *E. coli*, Streptococcus and Bacteroides and one strain of bifidobacteria caused more than 15% degradation of SAIB in 20 hr and a number of strains of *E. coli* and Lactobacillus produced less than 5% hydrolysis.

Human studies in vivo

The urinary excretion of sucrose and sucrose esters after a single oral dose of 0·1 or 1 g SAIB was less than the limit of detection (1 ppm as sucrose) of the assay procedure used. Therefore, of the 1 g SAIB administered, less than 0·4% was excreted in the urine as the parent compound or metabolites retaining the disaccharide moiety in any 24-hr period up to 5 days after the initial dose. A similar result was found for the urinary excretion of sucrose-containing compounds following seven daily doses of 1 g SAIB. No unchanged SAIB or metabolite was detected in faecal samples of the one subject given 0·1 g SAIB daily for 7 days.

The urinary excretion of sucrose following iv administration of 100, 250 and 500 mg sucrose is

Table 6. *Recovery in the urine of iv-administered sucrose*

Dose (mg)	Recovery* (%) at 3 hr	12 hr	Total recovery (%)
100	55·2	35·2	90·4
	83·3	12·0	95·3
250	49·0	18·9	67·9
	46·5	21·7	68·2
500	63·6	5·6	69·2

*No sucrose was detected (i.e. <1 mg/litre) in the urine at 24 hr at any dose level.
Values for 100- and 250-mg doses are the results of two separate experiments.

shown in Table 6. Approximately 50% of the administered compound was recovered in the urine after 3 hr at all three dose levels and there was almost quantitative recovery of the lowest dose after 12 hr.

DISCUSSION

We have shown that the extent of hydrolysis of SAIB by the intestinal contents of the rat declines progressively from the duodenum to the caecum, a finding which correlates with the gradual fall in esterase activity of the mucosa of the rat small intestine from the proximal to distal end reported by Harrison & Webster (1971). This suggests that the hydrolysis of SAIB in the intestinal tract of the rat is not dependent on the gut microflora, the numbers of which increase from the proximal ileum to the caecum (Drasar, Hill & Williams, 1970; Smith, 1965), but on non-specific esterases associated with the intestinal mucosa, homogenates of which were shown to hydrolyse SAIB rapidly. The finding that the extent of degradation of SAIB when introduced directly into the caecum of rats was less than that in the small intestine after oral dosing at a similar time interval supports this view.

Evidence for the lack of absorption of SAIB or partially esterified sucrose from the rat intestinal tract was provided by the *in vivo* experiments. Rats given [^{14}C]SAIB orally retained the majority of the radioactivity in the intestinal tract for at least 6 hr. During this time hydrolysis to low-molecular-weight esters was occurring, although the extent of complete hydrolysis to glucose and fructose, as shown by $^{14}CO_2$ excretion, was low. Absorption experiments using isolated loops of rat small intestine confirmed that the clearance of SAIB from the intestine was slow. After 24 hr a considerable proportion of the orally administered dose of [^{14}C]SAIB had been completely hydrolysed and utilized in physiological processes, as shown by $^{14}CO_2$ excretion, and retention of radioactivity in the body outside the gastro-intestinal tract at this time accounted for less than 5% of the administered dose.

In vitro experiments with human faecal suspensions and with pure cultures of a range of human intestinal micro-organisms suggest that, as in the rat, gut bacteria are not primarily responsible for the degradation of SAIB in the human alimentary tract. The lack of any detectable free sucrose in the urine of human volunteers given SAIB orally, suggests that the absorption of partially esterified sucrose molecules from the intestinal tract is negligible, as in the rat. Our results, and those of Weser & Sleisenger (1967), have shown that circulating sucrose, which would be the expected product of de-esterification of absorbed partial-esters of sucrose, is excreted substantially unchanged in the urine.

The data from these studies suggest that in man and in the rat, orally administered SAIB is hydrolysed in the intestinal tract to physiological components by non-specific esterases associated with the intestinal epithelium and is subsequently utilized extensively as a food source. These results, and other published data (Krasavage *et al.* 1973; Procter *et al.* 1973; Reynolds *et al.* 1974), suggest that the use of SAIB as a suspending agent in soft drinks is unlikely to present a toxicological problem in terms of its metabolic fate in man.

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LONG-TERM TOXICITY STUDIES OF SORBIC ACID IN MICE

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Abstract—Sorbic acid was given to groups of 48 male and 50 female mice at dietary levels of 0 (control), 1, 5 or 10% for 80 wk. Treatment had no adverse effect on the number of deaths or the incidence of histological lesions, including tumours. Compared with the controls, there were slightly lower body weights in mice given 10% sorbic acid, increased relative kidney weights at the two higher levels and increased relative liver weights at all treatment levels. The last finding and the isolated differences in the haematological examinations were not considered to represent adverse effects. It is concluded that dietary levels of sorbic acid up to 10% did not exert any carcinogenic effect and that the no-untoward-effect level was 1% of the diet.

INTRODUCTION

Sorbic acid (*trans,trans*-2,4-hexadienoic acid; $CH_3 \cdot CH{:}CH \cdot CH{:}CH \cdot COOH$) is used as a preservative in a wide range of foodstuffs. It may prevent spoilage of various foods when incorporated in the product, sprayed on the surface or included in the wrapper. It is particularly useful in inhibiting the growth of fungi and yeasts, and has some activity against bacteria.

Despite the low order of toxicity and the wide use of sorbic acid in other countries, the Food Additives and Contaminants Committee (1972) reviewed the situation and recommended that the permitted use of the additive in the UK should not be extended, as data from long-term studies were inadequate. The legislative position and data relating to the acute toxicity and metabolism of sorbic acid were reviewed by Gaunt, Butterworth, Hardy & Gangolli (1975). These authors also reported a long-term study in which no carcinogenic effect was detected in rats given sorbic acid at dietary levels up to 10%. The no-effect level was considered to be 1·5% of the diet, with changes of doubtful significance at the higher level (10%).

The present study in mice extends the long-term administration of sorbic acid to a second rodent species. In addition, as part of a series of investigations in our laboratories, long-term studies have been carried out in rats (Mason, Gaunt, Hardy, Kiss, Butterworth & Gangolli, 1976) and mice (Mason, Gaunt, Hardy, Kiss & Butterworth, 1976) comparing the effects of pure sorbic acid and sorbic acid deliberately adulterated with 1000 ppm parasorbic acid. The reason for these two studies was the concern expressed in the report of the Food Additives and Contaminants Committee (1972) relating to possible contamination of sorbic acid with parasorbic acid. In neither of these studies was there any indication of a carcinogenic effect on the part of parasorbic acid.

EXPERIMENTAL

Materials. The sorbic acid used in these studies was manufactured by Farbwerke Hoechst AG, Frankfurt, and complied with the specification prepared by the British Standards Institution (1967), according to which sorbic acid shall consist essentially of *trans, trans*-2,4-hexadienoic acid in the form of a near-white crystalline solid, with the characteristic odour and infra-red spectrum of sorbic acid, and shall have the following properties: sorbic acid content, min. 99·0%; 5 g dissolved in 100 ml acetone gives a clear or slightly opalescent solution, free of extraneous matter; melting range, 133–137°C; water content, max 0·5%; aldehyde content, max 0·15% (calculated as acetaldehyde) or max 0·1% (calculated as formaldehyde); arsenic, max 2 ppm; lead, max 10 ppm; sulphated ash, max 0·2%. Samples of sorbic acid from the same source as that used in the present studies were found to have a purity of 99·95 ± 0·05%. Corn oil was supplied by J. Sainsbury Ltd., London.

Animals and diet. Male and female mice of the ASH/CS1 strain were used. They were given free access to Oxoid pasteurized breeding diet supplemented with vitamin K_3 and to tap-water. The animals were housed in a room maintained at 20 ± 1°C with a relative humidity of 50–60%.

Experimental design and conduct. Groups of 48 male and 50 female mice were fed diets containing 0 (control), 1, 5 or 10% sorbic acid for 80 wk. The female mice were caged in groups of five and the male mice individually. In order to provide similar calorific intake in all groups, a mixture of corn oil and starch (1:1, w/w) was added to the 0, 1 and 5% sorbic acid diets at the rate of 10, 9 and 5% respectively.

Body weights of male mice were recorded individually at the beginning of the study and at intervals up to 74 wk. For the female mice, the combined weight of the inhabitants of each cage was recorded at the same intervals.

Ten or 20 mice of each sex from the control group and from those given the 5 or 10% sorbic acid were bled from the caudal veins at wk 13, 26 and 52. Blood was collected also from males given the 1% dietary level at wk 13 and from both sexes on this diet at wk 26. At the end of the study blood samples were collected from all surviving mice. All samples were

examined for haemoglobin concentration and slides were prepared for the enumeration of the various types of leucocytes, although examination of the latter was confined to the controls and the group on the highest dietary level, except at wk 13 when males of the 5% group were included. In addition, in the samples collected at wk 13, 26 and 52, the packed cell volume was measured and counts were made of erythrocytes, total leucocytes and reticulocytes.

Mice that showed signs of ill health were isolated, and were killed if they failed to recover. An autopsy was carried out on these mice and on those dying during the study unless this was prevented by advanced autolysis or cannibalism.

At the termination of the experiment, the remaining mice were killed by exsanguination from the aorta under barbiturate anaesthesia following an overnight period without food. At autopsy any macroscopic abnormalities were noted and the brain, heart, liver, spleen, kidneys, stomach and small intestine were weighed. Samples of these tissues, together with salivary gland, thyroid, thymus, adrenal glands, lymph nodes, pancreas, pituitary, ovaries and uterus or testes, urinary bladder, trachea, lungs, oesophagus, caecum, colon, rectum, skeletal muscle and any other tissue that appeared to be abnormal, were preserved in 10% neutral buffered formalin. Paraffin-wax sections of these tissues from all mice were stained with haematoxylin and eosin and examined microscopically.

RESULTS

In all groups of mice there were deaths during the study (Table 1). In both males and females the earliest deaths occurred in the treated mice, but there was no significant relationship between the dose level and the total number of deaths at any time.

Throughout the experiment the body weights were lower in the males fed 10% sorbic acid than in the controls (Table 2), the differences being statistically significant, and there was a similar, but non-significant, difference in the females from wk 17. The body weights at autopsy were significantly lower than the controls in the males given 5% sorbic acid and in males and females given 10% sorbic acid.

There were no statistically significant differences between treated female mice and the corresponding controls in the results of the haematological examinations (Table 3). In the male animals the results for all groups were in the same range during the last 6 months of the study. At the first examination (wk 13), the mean haemoglobin concentrations for all treated male groups were lower than those of the controls. There was no indication of a dose-relationship in the results and no comparable effects were seen in the values for the packed cell volume or erythrocyte count. Similarly the lower haemoglobin concentrations were not associated with a reticulocytosis. At wk 26 in the males there were isolated lower values for haemoglobin concentration (5% group) and erythrocyte count (1% group). The values at the higher levels were normal.

The weights of the hearts of the females given diet containing 5% sorbic acid showed a statistically significant increase compared with those of the controls (Table 4). The mean heart weight of the females given 1% sorbic acid was similarly higher, although the difference was not statistically significant in this case. The heart weight at the highest level did not differ from that of the controls. Liver weights of females on the 5 or 10% diets were increased compared with those of controls. In the males the spleen weights of all treated groups were less than those of the controls, with no indication of a dose-relationship. The weights of the kidneys of males given 5% sorbic acid were significantly greater than the control kidney weight. When the organ weights were expressed relative to

Table 2. *Mean body weights of mice fed diets containing 0–10% sorbic acid for 80 wk*

Dietary level (%)	Body weight (g) at wk						
	0	5	10	21	35	48	74
Males							
0	23	31	31	39	43	43	42
1	22	31	31	38	42	43	42
5	24	31	31	38	41	41*	40
10	23	30**	29***	35***	38**	37***	37***
Females							
0	18	25	27	32	36	37	37
1	17	25	27	32	36	37	36
5	17	25	26	31	34	35	37
10	17	25	27	31	33	34	33

The values are the means for all surviving mice (initially 48 in the male groups and 50 in the females). Those marked with asterisks differ significantly (Student's t test) from the corresponding controls: $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

Table 1. *Cumulative mortality in mice fed diets containing 0–10% sorbic acid for 80 wk*

Wk on test	Dietary level (%)...	Total no. of deaths							
		Males				Females			
		0	1	5	10	0	1	5	10
20		0	1	0	1	0	0	1	0
40		1	2	5	3	0	1	2	3
60		2	3	5	5	10	10	6	6
80		8(3)	10(6)	12(4)	9(4)	15(9)	20(10)	15(6)	16(10)

Figures represent the total numbers of mice found dead or killed *in extremis* from groups of 48 (males) or 50 (females). The values for treated mice did not differ significantly ($P = 0.05$ by chi-square test) from those for the controls. Numbers in parenthesis indicate the number of rats in each group killed *in extremis* during the study.

Table 3. *Results of haematological examinations in mice given diet containing 0–10% sorbic acid for 80 wk*

Sex and dietary level (%)	No. of mice examined	Hb (g/100 ml)	PCV (%)	RBC (10^6/mm^3)	Retics (% of RBC)	Leucocytes: Total (10^3/mm^3)	Leucocytes: Differential (%) N	E	L	M
Wk 13										
Male										
0	20	14·7	52	9·13	1·2	14·4	28	0	71	1
1	20	13·7*	50	8·59	—	11·8	—	—	—	—
5	20	13·8*	51	8·68	—	12·7	22	1	75	2
10	20	13·2**	52	9·11	1·3	12·9	24	1	74	1
Female										
0	10	16·1	52	8·44	2·4	12·8	16	4	79	1
5	10	16·0	50	8·58	—	11·8	—	—	—	—
10	10	15·4	51	7·66	2·0	12·1	15	2	83	0
Wk 26										
Male										
0	20	16·5	52	8·18	0·5	17·8	28	1	70	1
1	10	15·5	49	7·31*	—	14·6	—	—	—	—
5	10	14·2*	52	8·35	—	16·9	—	—	—	—
10	20	16·0	52	7·73	0·9	13·9	27	0	72	1
Female										
0	20	16·1	49	7·94	1·2	11·6	20	0	80	0
1	10	16·5	49	7·93	—	11·2	—	—	—	—
5	10	15·6	48	8·01	—	9·2	—	—	—	—
10	20	16·1	48	7·78	1·0	9·2	13	1	86	0

Hb = Haemoglobin PCV = Packed cell volume RBC = Red blood cells
Retics = Reticulocytes N = Neutrophils E = Eosinophils
L = Lymphocytes M = Monocytes

The figures are means for the numbers of mice shown and those marked with asterisks differ significantly (Student's *t* test) from those of the appropriate control: $*P < 0{\cdot}05$; $**P < 0{\cdot}01$. There were no statistically significant differences between test and control values at wk 52 and 80.

Table 4. *Organ weights of mice fed diet containing 0–10% sorbic acid for 80 wk*

Sex and dietary level (%)	No. of mice examined	Weight of: Brain	Heart	Liver	Spleen	Kidney	Stomach	Small intestine	Terminal body weight (g)
Organ weight (g)									
Male									
0	40	0·44	0·23	1·78	0·16	0·69	0·43	1·55	40·3
1	38	0·45	0·23	1·82	0·11*	0·70	0·42	1·59	39·3
5	36	0·44	0·23	1·91	0·11	0·76**	0·46	1·57	36·9***
10	39	0·44	0·21	1·76	0·11*	0·73	0·42	1·56	34·1***
Female									
0	35	0·44	0·17	1·62	0·19	0·48	0·50	1·48	34·3
1	31	0·46	0·19	1·78	0·21	0·49	0·49	1·42	33·5
5	35	0·45	0·19**	1·88**	0·18	0·49	0·50	1·38	35·1
10	31	0·46	0·17	1·86**	0·26	0·53	0·48	1·43	30·2**
Relative organ weight (g/100 g body weight)									
Male									
0	40	1·11	0·59	4·45	0·40	1·72	1·07	3·88	
1	38	1·16	0·59	4·69	0·28	1·74	1·09	4·09	
5	36	1·22**	0·63	5·24***	0·31	2·09***	1·26**	4·28*	
10	39	1·30***	0·63	5·14***	0·34	2·17***	1·25**	4·59***	
Female									
0	35	1·32	0·50	4·84	0·56	1·42	1·49	4·35	
1	31	1·42	0·56*	5·37*	0·63	1·51	1·52	4·30	
5	35	1·31	0·55*	5·43*	0·52	1·41	1·46	4·00*	
10	31	1·53***	0·57**	6·16***	0·83*	1·78**	1·59	4·85*	

Values are means for the numbers of mice shown and those marked with asterisks differ significantly (Student's *t* test) from the corresponding controls: $*P < 0{\cdot}05$; $**P < 0{\cdot}01$; $***P < 0{\cdot}001$.

body weight and compared with the control values, there were statistically significant increases in the values for brain, liver, kidney, stomach and small intestine in the males given 5 or 10% sorbic acid. In addition, the values for the relative heart and liver weights in all groups of females treated with sorbic acid, as well as those for the relative brain, small intestine, kidney and spleen weights in the females on the highest dietary level, were higher than those in the controls (Table 4).

The incidence of histological abnormalities is shown in Table 5. There were inflammatory or degenerative changes in kidney, lung, liver and urinary bladder. In the kidney the degenerative changes consisted of tubular dilatation with cast formation, increases in the numbers of interstitial cells and glomerular capsular adhesions. The degenerative changes in the liver were confined to the presence of scattered hepatocytes with a foamy appearance, and in two bladders the presence of chronic inflammatory cells was reported. The incidence of these lesions was in most cases less in the treated mice than in the controls. Amyloid was found in the liver, spleen and ovary of one female mouse given 10% sorbic acid. Benign hyperplastic nodules occurred in the livers of one male control mouse and of one female given 10% sorbic acid.

Table 6 summarizes the incidence and types of tumours found. Pulmonary adenomas occurred in 38% of all male mice and in 19% of all females. The incidence was similar in all groups in the males but was lower in the females given sorbic acid than in the female controls. Tubular adenomas of the kidney occurred only in male mice, with a similar incidence in controls and in animals treated with sorbic acid. The only tumours found in mice given sorbic acid without a comparable finding in the controls were a squamous epithelioma in a top-level female mouse, a squamous-cell carcinoma of the stomach in a male mouse given 1% sorbic acid and a single mammary adenocarcinoma in a female mouse given 10% sorbic acid.

DISCUSSION

It is possible that the reduction in weight gain in males and females given the highest dietary level was an indication of a slight toxic effect due to the intake of sorbic acid. In a long-term toxicity study in the rat, Gaunt *et al.* (1975) found a similar significant reduction in the rate of body-weight gain in both male and female rats given diets containing 10% sorbic acid. However, when the daily sorbic acid intakes of the two species are compared in relation to body weight, the mice given 10% sorbic acid in the diet received nearly three times as much of the compound as did the rats on the same diet. This may account for the fact that the 10% group of male mice showed a pronounced reduction of weight gain, since even at the 5% level mice received daily more than the mg/kg equivalent of rats given the 10% dietary level. Reduced palatability of the 5 and 10% supplemented diets cannot be excluded as a factor, since food consumption was not measured in the present study, but there was no firm evidence from our rat study (Gaunt *et al.* 1975) to suggest that the diets containing sorbic

Table 5. *Incidence of histopathological findings in mice fed diet containing 0–10% sorbic acid for 80 wk*

Organ and histological finding	Incidence of lesions							
	Males				Females			
Dietary level (%)...	0	1	5	10	0	1	5	10
No. of mice examined...	44	46	43	43	48	46	43	43
Kidney								
Perivascular lymphocytes	26	15*	12**	5***	29	9***	3***	6***
Degenerative changes	1	1	0	1	1	11**	3	2
Liver								
Early degenerative changes	15	3**	7	5*	4	4	4	6
Chronic inflammation	0	0	0	0	4	2	0	0
Hyperplastic nodules	1	0	0	0	0	0	0	1
Amyloid	0	0	0	0	0	0	0	1
Spleen								
Amyloid	0	0	0	0	0	0	0	1
Ovary								
Amyloid	—	—	—	—	0	0	0	1
Follicular cysts	—	—	—	—	7	3	1*	2
Lung								
Chronic inflammation	1	0	0	0	3	2	3	4
Thymus								
Hyperplasia	2	0	1	0	2	3	0	1
Bladder								
Chronic inflammation	0	2	0	0	0	0	0	0

Figures indicate the numbers of mice affected among the numbers examined and those marked with asterisks differ significantly (chi-square test) from those of the corresponding control: $*P < 0{\cdot}05$; $**P < 0{\cdot}01$; $***P < 0{\cdot}001$.

Table 6. *Incidence of tumours in mice fed diet containing 0–10% sorbic acid for 80 wk*

Organ and histological finding	Incidence of tumours							
	Males				Females			
Dietary level (%)...	0	1	5	10	0	1	5	10
No. of mice examined...	44	46	43	43	48	46	43	43
Lung								
Adenoma	19	16	16	15	14	5*	10	5*
Kidney								
Tubular adenoma	1	1	1	0	0	0	0	0
Testis								
Benign interstitial-cell tumour	1	0	0	0	—	—	—	—
Ovary								
Granulosa-cell tumour	—	—	—	—	1	1	0	0
Skin								
Squamous epithelioma	0	0	0	0	0	0	0	1
General								
Lymphoblastoma	4	3	2	3	4	4	3	5
Stomach								
Squamous-cell carcinoma	0	1	0	0	0	0	0	0
Mammary tissue								
Adenocarcinoma	0	0	0	0	0	0	0	1

Figures indicate the numbers of mice affected among the numbers examined and those marked with an asterisk differ significantly (chi-square test) from those of the corresponding controls: $*P < 0{\cdot}05$.

acid were unpalatable. In the past it has been suggested that feeding of dietary levels of sorbic acid up to 5% led to an increase in weight, possibly because of the calorific contribution of the acid (Deuel, Alfin-Slater, Weil & Smyth, 1954). This factor was controlled in the present study by the addition of starch and oil to the diet, and the dietary regimen adopted is therefore considered to be valid for detecting any adverse effects of sorbic acid on body weight. Since only the mice treated with the highest level of sorbic acid weighed significantly less than the controls and showed no other adverse effects, the lower weight may be considered to represent only a mildly unfavourable response.

The lower haemoglobin concentrations found in males after 3 months did not appear to be related to treatment. This conclusion is based on the observations that there were no parallel reductions in the other erythrocyte measurements and that the differences were confined to one sex. In addition, there was no significant dose-relationship in the magnitude of the effect, despite a tenfold difference in the dietary concentrations. Further evidence for a lack of direct effect of sorbic acid is the fact that the findings were not repeated at later examinations. These considerations, combined with the absence of any similar effect in rats (Gaunt *et al.* 1975), suggest that sorbic acid does not exert any effect on the haemopoietic system.

The analysis of the values for the organ weights and relative organ weights is complicated by the differences in body weight. The higher values for the relative weights of brain, spleen, stomach and small intestine were seen in the absence of any significant differences in the absolute weights and with no indication of any histological change. Such increases in relative weights are frequently encountered in animals, particularly rats, with low body weights (Feron, de Groot, Spanjers & Til, 1973) and are not considered to reflect a toxic effect of the material under study. The increased values for relative heart weights in the females in the present experiment are anomolous, in that there were no comparable changes in the males. It is possible that the increased value at the highest level may be a reflection of the lower body weight. There was no dose-relationship in the magnitude of the difference from the controls. In addition, the weight of the hearts in the female controls was slightly less than would be expected in mice of this size (Brantom, Gaunt & Grasso, 1973; Grasso, Hardy, Gaunt, Mason & Lloyd, 1974). The differences seen in relative heart weight are likely, therefore, to be a reflection of unusual controls and of the failure to gain weight at a normal rate rather than of any effect of sorbic acid.

The increase in relative liver weights cannot be attributed to differences in body weight since some higher values were found in the absolute weights despite the lower body weights. In addition, Feron *et al.* (1973) working with rats concluded that increased relative liver weights even in animals with low body weights should be considered as an effect of treatment. Increases in relative liver weight have been reported in rats given sorbic acid in the diet at levels of 5% (W. Kieckebusch, K. Lang and W. Griem, unpublished data 1967), 8% (Deuel *et al.* 1954) or 10% (Demaree, Sjogren, McCashland & Cosgrove, 1955; Gaunt *et al.* 1975). Only in the work of Gaunt *et al.* (1975) was there any indication of histological change; this consisted of a marginal increase in mild fatty change and focal necrosis and was attributed by the authors to increased intakes of fatty acid. In

the present study, there was a lower incidence of histological change in the livers of mice given sorbic acid than in the comparable controls, despite the high dose level and prolonged treatment. Hence it is considered that the increased relative liver weights were a reflection of an increase in metabolic demand rather than a toxic effect of sorbic acid.

The increased relative kidney weights in mice given 5 or 10% sorbic acid cannot be accounted for wholly in terms of the decreased body weights and, as with the liver, Feron *et al.* (1973) consider that such increases should be attributed to treatment. The histological examination showed that, in general, the incidence of lesions in the kidney was significantly less in the treated mice than in the controls. On this basis the enlarged kidney does not represent any marked toxic effect on the part of sorbic acid. This finding is in keeping with the observations of Gaunt *et al.* (1975) in rats exposed to 10% sorbic acid for 2 yr.

Most of the types of tumour encountered occurred with a similar or higher frequency in control than in treated mice, and therefore provided no indication of any effect on the part of sorbic acid. The tumours occurring without comparable findings in the control animals in this study cannot be attributed to the feeding of sorbic acid. The single malignant squamous epithelioma of the skin, although found in a female mouse on the highest dietary level, was a single observation among 264 treated animals and cannot be construed as a carcinogenic effect since such tumours are known to occur spontaneously (Cloudman, 1956). The single mammary adenocarcinoma in a female mouse fed 10% sorbic acid represents an incidence of 0·56% of the total females examined histopathologically. This is similar to the overall incidence (0·6–0·8%) recorded in females of this strain of mice at the end of other studies in these laboratories (Brantom *et al.* 1973; Grasso *et al.* 1974; Mason, Gaunt, Butterworth, Hardy, Kiss & Grasso, 1974).

In our experience the isolated squamous-cell carcinoma of the stomach in a male mouse given 1% sorbic acid is an uncommon type of tumour in ASH/CS1 mice. Only one squamous-cell carcinoma, occurring in the anogenital region of a male mouse given a diet containing a low dose of a non-carcinogenic material, has been found in our laboratory (P. L. Mason, I. F. Gaunt, K. R. Butterworth & J. Hardy, unpublished report 1974). Nevertheless, this single occurrence in the group on the low dietary level in the present experiment cannot be taken to indicate a carcinogenic effect.

From the above data it is clear that sorbic acid fed at levels up to 10% of the diet of mice for 80 wk did not exhibit any signs of carcinogenicity. No adverse effects, other than a slight enlargement of the kidney and a reduction in body-weight gain, were seen in mice fed 5 or 10% dietary sorbic acid, and these changes were not observed at the 1% dietary level. On this basis the no-untoward-effect level may be considered to be 1% of the diet (providing an intake of approximately 1400 mg/kg/day). This is higher than the value of 750 mg/kg/day established by Gaunt *et al.* (1975) in a similar study in rats. In view of the nature of the findings at levels up to 10% it is likely that the true no-untoward-effect level may be considerably higher.

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LONG-TERM TOXICITY OF PARASORBIC ACID IN RATS

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Abstract—Sorbic acid and sorbic acid containing 1000 ppm parasorbic acid were given at a dietary level of 1·2% to groups of 48 male and 48 female rats for 2 yr. The inclusion of parasorbic acid had no significant effect on body-weight gain, intakes of food or water, haematological examinations, renal function tests, serum analyses or the findings of the histopathological examination, including the incidence of tumours. The liver weight and relative liver weight of female rats given parasorbic acid in the diet were increased compared with those of animals given sorbic acid alone, but this finding is considered to be of doubtful significance. It is concluded that the inclusion of 1000 ppm parasorbic acid does not adversely influence the outcome of feeding sorbic acid to rats over prolonged periods.

INTRODUCTION

Sorbic acid (*trans,trans*-2,4-hexadienoic acid) is a preservative permitted for use in food in the UK (The Preservatives in Food Regulations 1962, Statutory Instrument 1962, no. 1532), in the USA (Sec. 121.101 of the Code of Federal Regulations) and in a number of other countries. In the UK, this use of sorbic acid was reviewed by the Food Additives and Contaminants Committee (1972) and its report recommended that the use of this preservative should not be extended because of the inadequacy of the existing data from long-term studies. This report also suggested a limit of 1000 ppm on the level of parasorbic acid in samples of sorbic acid to be used for food-additive purposes.

The available data on the metabolism and toxicity of sorbic acid were reviewed by Gaunt, Butterworth, Hardy & Gangolli (1975), who fed diets containing 0, 1·5 and 10% sorbic acid to rats for 2 yr with no evidence of carcinogenic potential. The only effects encountered were increased relative liver and kidney weights at the highest level, together with increased thyroid weights in the males. It was concluded that the no-untoward-effect level in rats was 1·5% of the diet.

Particular concern has arisen over the possible contamination of sorbic acid with parasorbic acid (5-hydroxy-2-hexanoic acid δ-lactone), which is a naturally occurring lactone found in the berries of the species *Sorbus aucuparia* L. and from which sorbic acid was at one time derived by acid hydrolysis. This method of manufacture of sorbic acid has been superseded by a synthetic process involving catalytic condensation of crotonaldehyde with either ketene or malonic acid. It was considered by the staff of the Laboratory of the Government Chemist (Ministry of Technology: Laboratory of the Government Chemist, 1970) that parasorbic acid was unlikely to occur as a by-product of this process and that the ready conversion of parasorbic to sorbic acid made it unlikely that the former would be formed in pure sorbic acid. The conversion to parasorbic acid in preserved food, particularly during cooking, is more speculative.

Since parasorbic acid, like a number of other α,β-unsaturated lactones, has been shown to produce local sarcomas following repeated sc injection in rats (Dickens & Jones, 1961 & 1963), there was concern about the possible contamination of food with a putative carcinogen. In view of the lack of any consistent structure–activity relationship within this group of chemicals, it was suggested (Grasso, Gangolli & Hooson, 1969) that factors other than chemical carcinogenicity might be responsible for the production of the sarcomas. This conclusion was supported, in the case of parasorbic acid, by a failure to establish any evidence of carcinogenic potential in rats given drinking-water containing 0·2 or 1·0 mg parasorbic acid/100 ml for 64 wk (Dickens, Jones & Waynforth, 1966).

Much of the controversy over the possible contamination of sorbic acid with parasorbic acid has arisen as the result of inadequate methods for the detection of the latter compound. However, Murphy & Wardleworth (1973) described a method capable of detecting 0·5 mg parasorbic acid/kg sorbic acid, involving extraction from aqueous potassium sorbate with dichloromethane and subsequent determination by gas chromatography. By this method, no parasorbic acid was detected in the samples of sorbic acid analysed.

In view of the potential importance of sorbic acid as a preservative and the lack of data concerning it and parasorbic acid, a series of long-term experiments was initiated as part of the BIBRA safety evaluation programme. In addition to the study reported here, this programme involved feeding sorbic acid to rats for 2 yr (Gaunt *et al.* 1975) and parallel studies on sorbic acid (Hendy, Hardy, Kiss, Gaunt & Butterworth, 1976) and parasorbic acid (Mason,

Gaunt, Hardy, Kiss & Butterworth, 1976) in the mouse.

Since the problems associated with parasorbic acid arose as a consequence of its possible presence in sorbic acid, it was decided to study its effects by comparing the results obtained with deliberately contaminated sorbic acid and with the uncontaminated material. The dietary level of sorbic acid used was similar to that shown by Gaunt *et al.* (1975) to be without effect in comparison with untreated controls and the level of contamination of the sorbic acid (1000 ppm) was the maximum allowable level proposed by the Food Additives and Contaminants Committee (1972).

EXPERIMENTAL

Materials. The sample of parasorbic acid used in this experiment was synthesized in the Laboratory of the Government Chemist, while the sorbic acid was provided by Farbwerke Hoechst AG and complied with the specification of the British Standards Institution (1967), according to which the material shall consist essentially of *trans.trans*-2,4-hexadienoic acid in the form of a near-white crystalline solid with the characteristic odour and infra-red spectrum of sorbic acid and shall have the following properties: sorbic acid content, min. 99·0%; 5 g dissolved in 100 ml acetone gives a clear or slightly opalescent solution, free of extraneous matter; melting range, 133–137°C; water content, max 0·5%; aldehyde content, max 0·15% (calculated as acetaldehyde) or max 0·1% (calculated as formaldehyde); arsenic, max 2 ppm; lead, max 10 ppm; sulphated ash, max 0·2%. The sample used was from the same source as that used in the previous studies (Gaunt *et al.* 1975) and analysis of batches from this supplier had shown a purity of 99·5 ± 0.05%.

Animals and diet. Rats of a Wistar-derived strain obtained from a specified-pathogen-free breeding colony were housed, four to a cage, in a room maintained at 20 ± 1°C with a relative humidity of 50–60%. The animals were fed a basic diet of Spillers' Laboratory Small Animal Diet *ad lib.* and were allowed unlimited access to water.

Experimental design and conduct. Groups of 48 male and 48 female rats were fed diets containing 1·2% sorbic acid (SA group) or 1·2% sorbic acid containing 1000 ppm parasorbic acid (PSA group) for 2 yr. Body weight, food consumption and water consumption were measured initially and at intervals during the study. Blood samples, collected from a caudal vein of ten male and ten female rats from each group at wk 13, 23, 54 and 80 or taken during autopsy from the aorta of all rats surviving for 2 yr, were used for haematological examinations. Measurements were made of the haemoglobin concentration and packed cell volume together with counts of total erythrocytes, reticulocytes and total leucocytes. The red cell morphology was examined and the proportion of the different types of leucocytes was determined.

Urine samples, from ten rats of each sex from both groups, were examined for the presence of glucose, bile, blood, albumin and ketones during wek 12, 24 and 52. In addition, using the same rats, the urinary cell excretion and the volume and specific gravity of the urine were measured in samples collected over a 2-hr period following a water load of 25 ml/kg. A concentration test was conducted, involving measurements of the specific gravity and volume of the urine produced during a 6-hr period of water deprivation and a 4-hr period commencing after water deprivation for 16 hr. Serum samples collected from the rats surviving at 2 hr were analysed for levels of urea, total protein and albumin and for the activities of glutamic–oxalacetic transaminase, glutamic–pyruvic transaminase and lactic dehydrogenase.

During the study, the animals were kept under continual surveillance for any abnormalities in condition or behaviour. In the event of illness, the rats were isolated, to be killed if their condition deteriorated, or returned to their cage if the condition improved. An autopsy was carried out on all animals dying or killed during the study, unless this was precluded by autolysis or cannibalism. At the end of the experiment, all surviving rats were killed by exsanguination under barbiturate anaesthesia following an overnight period without food. At post-mortem examination, macroscopic abnormalities were noted and the brain, heart, pituitary, liver, spleen, kidneys, stomach, small intestine, caecum, gonads and adrenals were weighed. Samples of these tissues and of salivary gland, thymus, cervical and mesenteric lymph nodes, pancreas, urinary bladder, aorta, lung, trachea, colon, rectum, skeletal muscle, spinal cord and uterus, together with any other tissue that appeared to be abnormal, were preserved in 10% buffered formalin. Paraffin-wax sections of these tissues were stained with haematoxylin and eosin for histopathological examination.

RESULTS

There were no differences between the two groups in the appearance or behaviour of the rats and in these respects both groups were considered to be comparable with untreated rats maintained under the same conditions. The mortality was slightly greater in females of the PSA group than of the SA group, the figures being 33 and 21%, respectively, at the end of the experiment. This difference was largely attributable to five rats that died or were killed in the period

Table 1. *Cumulative mortality of rats fed diets containing 1·2% sorbic acid or 1·2% sorbic acid contaminated with 1000 ppm parasorbic acid for 2 yr*

Wk of treatment	Total no. of animals dead			
	Males		Females	
	SA	PSA	SA	PSA
40	1	1	0	0
60	2	1	0	1
80	4	6	1	5
90	11	11	6	8
100	22	17	9	14
106	24 (9)	20 (11)	10 (5)	16 (10)

SA = Group fed diet containing 1·2% sorbic acid
PSA = Group fed diet containing 1·2% sorbic acid contaminated with 1000 ppm parasorbic acid
The figures represent the total number of rats dead from groups of 48; those in parenthesis represent the number of rats in each group killed *in extremis*.

Table 2. *Body weight, food consumption and water consumption of rats fed diets containing 1·2% sorbic acid or 1·2% sorbic acid contaminated with 1000 ppm parasorbic acid for up to 2 yr*

Sex and group	Value at wk										
	0*	1	4	14	23	37	52	64	76	90	100
Body weights (g)											
Male											
SA	81	140	282	454	568	636	688	737	746	745	697
PSA	81	138	281	458	557	602	631	678	679	712	634
Female											
SA	68	114	178	249	294	325	354	390	412	442	452
PSA	70	115	184	259	306	335	363	402	426	475	460
Food consumption (g/rat/day)											
Male											
SA	13·0	17·8	21·7	21·6	23·7	21·0	19·8	19·1	18·8	20·6	20·2
PSA	11·4	18·4	21·7	23·8	23·3	21·2	19·7	19·0	28·1	20·2	19·9
Female											
SA	10·7	15·1	14·9	19·7	17·5	18·6	17·0	15·8	17·1	18·5	18·7
PSA	11·1	15·5	14·5	19·9	18·5	18·7	17·4	16·0	17·6	19·9	19·7

SA = Group fed diet containing 1·2% sorbic acid
PSA = Group fed diet containing 1·2% sorbic acid contaminated with 1000 ppm parasorbic acid
*First day of treatment.
Figures are means for all surviving rats in the case of body weight and for 12 cages each of four rats for food consumption.

between wk 58 and wk 80, the death rate in the subsequent period being similar in the two groups (Table 1). There were no comparable differences in the males, the overall mortality being 50 and 42% in the SA and PSA rats, respectively.

The body weight of the male PSA rats was less than that of the SA group from wk 23 onwards (Table 2) but the difference did not reach statistical significance and did not exceed 9% of the weight of the latter group. No differences between the two groups were seen in food consumption (Table 2) or in the intake of water. The intakes of sorbic acid were similar in the two groups, the daily average over the experiment being 0·41–0·45 g/kg in males and 0·62–0·63 in females. The corresponding figures for parasorbic acid intake in the PSA group were 0·1% of these values, or approximately 0·4 and 0·6 mg/kg/day in males and females, respectively.

Table 3. *Haematological findings in rats fed diets containing 1·2% sorbic acid or 1·2% sorbic acid contaminated with 1000 ppm parasorbic acid for up to 2 yr*

Sex and group	No. of animals examined	Hb (g/100 ml)	PCV (%)	RBC (10^6/mm^3)	Retics (% RBC)	Leucocytes				
						Total (10^3/mm^3)	Differential (%)			
							N	E	L	M
Wk 13										
Male										
SA	10	15·8	49	7·71	1·1	12·55	12	0	88	0
PSA	10	15·4	50	8·47**	0·8	13·00	12	0	88	0
Female										
SA	10	15·8	48	6·83	1·3	8·27	9	0	91	0
PSA	10	15·9	49	5·59***	1·1	8·82	8	1	91	0
Wk 104										
Male										
SA	22	14·4	41	5·94	1·3	5·21	35	2	61	2
PSA	28	14·7	42	5·92	1·1	4·63	33	1	65	1
Female										
SA	38	14·8	44	6·85	1·4	3·10	—	—	—	—
PSA	29	14·3	43	6·24*	1·3	2·72	—	—	—	—

Hb = Haemoglobin PCV = Packed cell volume RBC = Red blood cells
Retics = Reticulocytes N = Neutrophils E = Eosinophils L = Lymphocytes M = Monocytes
SA = Group fed diet containing 1·2% sorbic acid
PSA = Group fed diet containing 1·2% sorbic acid contaminated with 1000 ppm parasorbic acid
The figures are means of values for the numbers of rats shown and those marked with asterisks differ significantly (Student's t test) from those of the appropriate control: *$P < 0·05$; **$P < 0·01$; ***$P < 0·001$.

Table 4. *Organ weights and relative organ weights of rats fed diets containing 1·2% sorbic acid or 1·2% sorbic acid contaminated with 1000 ppm parasorbic acid for 2 yr*

Sex and group	No. of rats examined	Body weight (g)	Weight of										
			Brain	Heart	Liver	Spleen	Kidney	Stomach	Small intestine	Caecum	Adrenal†	Gonad‡	Pituitary†
			Organ wt (g)										
Male													
SA	22	672	2·13	1·81	17·34	1·40	4·92	2·88	9·85	1·46	87	3·64	12·9
PSA	29	640	2·12	1·62**	15·82*	1·32	4·49	2·82	9·79	1·51	76	3·51	11·3*
Female													
SA	39	435	1·88	1·13	10·00	0·87	2·30	2·09	6·89	1·19	78	168	16·3
PSA	31	426	1·89	1·20	11·83**	0·92	2·52	2·18	7·58*	1·30	85	251	17·1
			Relative organ weight (g/100 g body weight)										
Male													
SA	22	—	0·32	0·28	2·61	0·21	0·74	0·44	1·48	0·22	13	0·55	1·9
PSA	29	—	0·34	0·26	2·52	0·21	0·73	0·46	1·56	0·24	12	0·57	1·9
Female													
SA	39	—	0·45	0·26	2·31	0·20	0·53	0·49	1·62	0·28	18	40	3·7
PSA	31	—	0·45	0·29*	2·81***	0·22***	0·61*	0·52	1·83	0·31*	21*	60	4·0

SA = Group fed diet containing 1·2% sorbic acid

PSA = Group fed diet containing 1·2% sorbic acid contaminated with 1000 ppm parasorbic acid

†Values for weights and relative weights of this organ are expressed in mg and mg/100 g body weight, respectively.

‡Values for weight and relative weight of female gonads are expressed in mg and mg/100 g body weight respectively.

Figures are means of values for the numbers of rats shown and those marked with asterisks differ significantly (Student's *t* test) from those of controls: $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

Table 5. *The incidence of histopathological lesions in rats fed diets containing 1·2% sorbic acid or 1·2% sorbic acid contaminated with 1000 ppm parasorbic acid for 2 yr*

	Incidence of lesions			
	Male		Female	
	SA	PSA	SA	PSA
Organ and lesion No. of rats examined...	36	42	45	45
Liver				
Cell vacuolation	29	24	22	20
Bile-duct proliferation	13	4	13	1
Areas of necrosis or infarction	1	1	0	0
Foci of leucocytes	4	2	0	1
Extensive necrosis and inclusion bodies	0	1	0	0
Extramedullary haemopoiesis	0	1	1	0
Haemangioma	0	1	3	2
Bile cysts	2	1	1	0
Kidney				
Glomerulonephrosis	36	41	37	41
Mineral deposits	0	0	5	2
Lungs				
Emphysema	8	15	7	0
Thickened alveolar walls	5	3	1	0
Alveoli with foamy macrophages	9	9	10	4
Calcification of alveolar walls	5	6	0	1
Heart				
Fibrosis	28	26	4	3
Calcified deposits	3	3	0	2
Medial hypertrophy of coronary arteries	2	2	0	0
Spleen				
Extramedullary haemopoieses	11	4	12	14
Haemosiderin	8	8	19	15
Uterus				
Cystic	—	—	5	3
Cystic endometrial hyperplasia	—	—	2	4
Hyperplasia of uterine wall	—	—	1	1
Fibroid polyps	—	—	1	2
Ovaries				
Cystic	—	—	5	5
Testis				
Increase in amorphous interstitial substance	15	12	—	—
Increase in interstitial cells	1	1	—	—
Periarteritis	11	13	—	—
Calcified tubules	3	2	—	—
Atrophic tubules	1	5	—	—
One testis atrophic	3	4	—	—
Both testes atrophic	2	3	—	—
Adrenals				
Vacuolated cortical cells	13	16	9	7
Haemorrhage	6	3	16	16
Thyroid				
Numerous small follicles	10	5	2	0
Degenerating colloid	2	2	1	0
Large follicles	0	1	1	1
Atrophic follicles	0	1	1	0
Parathyroids				
Hyperplasia	3	6	0	1
Pituitary				
Haemorrhage	3	6	1	2
Hyperplasia	4	2	3	10
Stomach				
Dilated glands	16	16	13	8
Calcification of mucosa	1	5	0	1
Erosion of mucosa	0	1	2	0

Table 5—*continued*

		Incidence of lesions			
		Male		Female	
		SA	PSA	SA	PSA
Organ and lesion	No. of rats examined...	36	42	45	45
Mesentary					
Periarteritis		4	3	2	0
Calcified vessel		1	1	0	0
Brain					
Calcified foci		1	1	0	0

SA = Group fed diet containing 1·2% sorbic acid
PSA = Group fed diet containing 1·2% sorbic acid contaminated with 1000 ppm para-sorbic acid
The figures represent the numbers affected by the histopathological lesions out of the numbers of rats examined.

No abnormal constituents were found in the urine and the results of the renal concentration tests and urinary cell excretion showed no differences between the two treatments. Similarly there were no differences between the two groups in the results of the serum analyses.

There were some differences between the groups in the results of the haematological examination (Table 3) but these were not consistent. At wk 13, the erythrocyte count of the PSA group was lower than that of the SA group in females but higher in males. The animals were bled again 2 wk later when the erythrocyte counts in males were 8·0 and $7{\cdot}33 \times 10^6/mm^3$ for the SA and PSA groups, respectively. The corresponding values in females were 7·26 and $7{\cdot}14 \times 10^6/mm^3$. The differences between these figures were not statistically significant. The remaining differences between the groups occurred in females, in which, compared with the SA rats, the haemoglobin concentrations of the PSA rats were higher at wk 52 (by 7·5%) and at wk 80 (by 6·4%). At 2 yr, the erythrocyte count of this same group was lower by approximately 9%.

Compared with the SA rats, the weights of heart, liver and pituitary were lower in the male PSA rats to a statistically significant degree and the livers and small intestines in the females of the same group were significantly heavier (Table 4). When the organ weights were expressed relative to body weight, the values for heart, liver, kidney, spleen, caecum and adrenal glands were greater in the female PSA rats than in those of the SA group (Table 4).

The histopathological lesions encountered during the study were evenly distributed between the two treatment groups (Table 5) and most of the tumours found during the study occurred either in the SA group alone or with a similar frequency following either treatment (Table 6). There was a slightly higher incidence of adenomas of the pituitary in both sexes on the PSA diet than in those on the SA diet. This increase was not statistically significant, nor was the slight increase in mammary adenomas. The only benign tumours found solely in the PSA-treated rats were two papillary cystadenomas of the thyroid and an adenoma of the exocrine pancreas. In addition there were malignant tumours of the liver, uterus, mammary gland, skin and subcutaneous tissue in the PSA group. A fibrosarcoma originating from the cervix was found in a PSA female rat, while single cases of thymic lymphosarcoma and adrenal neuroblastoma were found only in SA rats. Three mammary adenocarcinomas were found, one in a female SA rat and two in female PSA rats. Malignant tumours of the skin, one keratoacanthoma and a squamous-cell carcinoma were encountered in a male SA rat and a PSA female, respectively. Two reticulum-cell neoplasms were found in PSA female rats, one in the liver with spread to the lymph nodes and the other in the ileum. A fibrosarcoma and sarcoma of the subcutaneous tissue were found in a male and a female rat respectively, both from the PSA group.

DISCUSSION

The decreases in heart, liver and pituitary weights in the male PSA rats are related to the slightly lowered body weight in these animals as evidenced by the disappearance of any significant differences when the weights were expressed relative to body weight. The observation of increased liver weight and relative liver weight in the females of the PSA group is anomalous in view of the tendency in the opposite direction in the males. Although it is possible that the difference resulted from the induction of sex-dependent enzyme systems within the liver, the small magnitude of the effect, its absence from the male animals and the lack of histopathological sequelae, despite lifetime feeding, indicate that it was not toxicologically significant. The increases in small-intestine weight and relative caecum weight in the female PSA rats occurred without any histopathological changes or any noticeable change in gastro-intestinal function, as judged by food intake and weight gain. On this basis the differences in weight were regarded as unrelated to treatment. Similarly the higher values for relative heart, spleen, kidney and adrenal weights were not associated with any particular histopathological changes in these organs. The differences repre-

Table 6. *Incidence of tumours in rats fed on diets containing 1·2% sorbic acid or 1·2% sorbic acid contaminated with 1000 ppm parasorbic acid for 2 yr*

Organ and type of tumour	Incidence of tumours			
	Male		Female	
	SA	PSA	SA	PSA
No. of rats examined...	36	42	45	45
Liver				
Reticulum-cell neoplasm	0	0	0	1
Lipoma	2	0	0	0
Kidney				
Lipoma	0	0	1	0
Lungs				
Adenoma	0	0	1	0
Testis				
Interstitial-cell tumour	1	1	—	—
Uterus				
Fibrosarcoma	—	—	0	1
Fibroma	—	—	1	1
Mammary gland				
Fibroadenoma	—	—	6	13
Adenocarcinoma	—	—	1	2
Skin				
Keratoacanthoma	1	0	0	0
Squamous-cell carcinoma	0	0	0	1
Pituitary				
Chromophobe adenoma	7	10	11	14
Thyroid				
Adenoma	1	3	1	2
Papillary cystadenoma	0	1	0	1
Adrenals				
Neuroblastoma	1	0	0	0
Medullary tumour	2	1	0	0
Pancreas				
Islet-cell tumour	1	0	1	0
Exocrine adenoma	0	1	0	0
Lipoma	0	0	1	0
Ileum				
Reticulum-cell neoplasm	0	0	0	1
Mesentary				
Lipoma	0	0	1	0
Salivary gland				
Adenoma	1	0	0	0
Thymus				
Lymphosarcoma	0	0	1	0
Subcutaneous tissue				
Myoma	0	1	0	0
Leiomyoma	1	0	0	0
Fibroma	1	1	0	0
Fibrosarcoma	0	1	0	0
Sarcoma	0	0	0	1

SA = Group fed diet containing 1·2% sorbic acid
PSA = Group fed diet containing 1·2% sorbic acid contaminated with 1000 ppm parasorbic acid
The figures show the numbers of rats affected among the numbers of rats examined.

sented 10–15% of the value for the SA group and, although not significantly different, the remainder of the relative organ weights of the PSA group were higher by a similar amount. Such a consistent increase in all of the relative organ weights suggests some common factor, for example an influence of body weight, rather than an effect of treatment on the particular organs.

The differences in the numbers of red blood cells at wk 12 were inconsistent in the direction of change in the two sexes. In addition, when counts were carried out 2 wk later as a check, there were no significant differences, a result suggesting a fortuitous finding in the first samples. These changes, like the lower red cell count at wk 104 in the PSA females, were not accompanied by any change in haemoglobin concentration, packed cell volume or reticulocyte count. This lack of other indications of increased red-cell turnover or of decreased red-cell production suggests that the altered erythrocyte counts were a chance occurrence within the normal range and were unrelated to treatment.

There was no evidence to implicate parasorbic acid in the formation of any of the histopathological lesions, including tumours, found during the study. The commonest tumours—mammary fibroadenomas and pituitary adenomas—are both well-known spontaneous tumours in rats (Snell, 1965). Indeed this author quotes incidences as high as 68% for pituitary adenomas in female Wistar rats. In addition, both types of lesion have been found with a frequency similar to or greater than that seen in the present study in untreated rats of this strain in our laboratories (Brantom, Gaunt, Hardy, Grasso & Gangolli, 1973), including the controls in an experiment with sorbic acid (Gaunt *et al.* 1975). Such a spontaneously high and variable incidence of these tumours precludes any suggestion that the slight increases of incidence in the PSA rats might be due to treatment.

The majority of the remaining tumours were found either in SA animals alone or with a similar incidence in both groups, again precluding an effect of parasorbic acid treatment. The tumours found only in animals given parasorbic acid were present in the liver, uterus, skin, thyroid, pancreas, ileum and subcutaneous tissue. Apart from adenomas of the thyroid and exocrine pancreas, and a myoma of the subcutaneous tissue, all of these tumours were malignant. However, without exception, they have been described in untreated rats (Snell, 1965) and have been observed in previous studies using this strain of rat (Brantom *et al.* 1973; Gaunt *et al.* 1975) and their occurrence in the present experiment cannot be taken as evidence of any carcinogenic effect of parasorbic acid.

The results of this study have shown that the feeding to rats of sorbic acid contaminated with 1000 ppm parasorbic acid does not increase the toxic potential of the sorbic acid or show any evidence of a carcinogenic potential when compared with treatment with sorbic acid at a level that has been shown to be without effect (Gaunt *et al.* 1975).

Lishmund (1969) calculated that if sorbic acid were used in a wide range of foodstuffs, including bread, the average daily intake would be 293 mg. If this amount contained parasorbic acid at the maximum level (1000 ppm) suggested by the Food Additives and Contaminants Committee (1972), the intake of the lactone would be approximately 0·3 mg/day. The no-untoward-effect level from the present study is at least 0·4–0·6 mg/kg/day, which after application of the traditional 100-fold safety factor would indicate an acceptable intake of 0·24–0·36 mg/day for a 60-kg adult. Therefore there is no evidence to suggest a harmful effect from the prolonged ingestion of parasorbic acid at approximately 100 times the likely maximum intake by man. Additionally it is unlikely that such intakes would be reached, since parasorbic acid was not present at the detectable level of 0·5 ppm in the samples of sorbic acid analysed (Murphy & Wardleworth, 1973).

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LONG-TERM TOXICITY OF PARASORBIC ACID IN MICE

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Abstract—Groups of 48 male and 48 female mice were fed for 80 wk on diet containing either 1·2% sorbic acid or 1·2% sorbic acid deliberately adulterated with 1000 ppm of specially prepared parasorbic acid. There were no statistically significant differences between the two treatments in body-weight gain, haematological examinations, organ weights or the incidences of histopathological findings, including tumours. Mortality was slightly higher in females given diet containing parasorbic acid than in those given only sorbic acid, but this difference was not considered to be related to treatment. It is concluded that the inclusion of parasorbic acid at a level of 1000 ppm in sorbic acid did not lead to an increase in the toxic effects of the latter, nor did the results suggest a carcinogenic effect.

INTRODUCTION

Sorbic acid (*trans,trans*-2,4-hexadienoic acid) is widely used as a preservative in food. The data relating to its toxicity were reviewed by Gaunt, Butterworth, Hardy & Gangolli (1975), who established that there was no evidence of carcinogenic potential when sorbic acid was fed to rats at a dietary level of 10% for 2 yr. They concluded that the no-effect level was 1·5% of the diet. Similarly, Hendy, Hardy, Kiss, Gaunt & Butterworth (1976) found no evidence of a carcinogenic effect when sorbic acid was fed to mice for 80 wk at dietary levels up to 10%.

Nevertheless, as pointed out by Mason, Gaunt, Hardy, Kiss, Butterworth & Gangolli (1976), concern has arisen concerning the possible contamination of sorbic acid with parasorbic acid (5-hydroxy-2-hexanoic acid δ-lactone) to the extent that it was suggested, in the report of the Food Additives and Contaminants Committee (1972), that there should be a limit of 1000 ppm for parasorbic acid in sorbic acid. Mason *et al.* (1976) reviewed the available data relating to the toxicological effects of parasorbic acid and reported a study showing no adverse effects in rats that consumed for 2 yr a diet containing 1·2% sorbic acid deliberately adulterated with 1000 ppm parasorbic acid compared with animals consuming the same level of pure sorbic acid. The present paper contains the results of a similar long-term study in mice carried out as part of the BIBRA safety evaluation programme.

EXPERIMENTAL

Materials. The parasorbic and sorbic acids used in this study were the same as those used in the 2-yr rat study (Mason *et al.* 1976).

Animals and diet. The mice used in this study were of the ASH CS1 strain, obtained from a specified-pathogen-free colony, and were housed in a room maintained at 20 ± 1°C with a relative humidity of 50–60%. They were fed a basic diet consisting of reground Oxoid pasteurized breeding diet, supplemented with 40 ppm sodium menadione bisulphite, and had unlimited access to water.

Experimental design and conduct. Groups of 48 male and 48 female mice were fed diets containing either 1·2% sorbic acid (referred to as the SA group) or 1·2% sorbic acid containing 1000 ppm parasorbic acid (the PSA group). The female mice were caged in groups of four and the male mice were caged individually. Body weights of all animals were recorded initially and at intervals throughout the study. The animals were under continual surveillance, and any animal that appeared to be ill and unlikely to survive was killed.

A post-mortem examination was made on these mice and on any that died during the experiment, unless this was precluded by advanced autolysis or cannibalism. Animals surviving to wk 80 were killed by exsanguination under barbiturate anaesthesia, all organs were examined for macroscopic changes and the brain, heart, liver, spleen, kidneys, stomach and small intestine were weighed. Samples of these organs, together with samples of salivary gland, thyroid, thymus, adrenals, lymph nodes, pancreas, pituitary, uterus, urinary bladder, lungs, gonads, small intestine, caecum, colon, rectum, spinal cord, skeletal muscle and any other tissue that appeared abnormal at autopsy were preserved in 10% buffered formalin. Paraffin-wax sections of these tissues were stained with haematoxylin and eosin and examined for histopathological changes.

Blood samples were collected from the caudal veins of ten mice of each sex from both groups at wk 12, 26 and 51 as well as from all surviving animals immediately before killing at wk 80. Measurements were made of haemoglobin and packed cell volume together with erythrocyte and leucocyte counts. Slides were prepared for the enumeration of reticulocytes and the different types of leucocytes but, in the absence of any effects in the other measurements, these were not examined.

RESULTS

The death rates in both groups of male animals were similar (Table 1). In the females, however, the first deaths occurred earlier in the study in the PSA mice, with five animals dead from this group at wk 60, before the first animal had died in the SA group. This trend continued, so that during the last 3 wk of the study the total number of mice dead was significantly greater ($P < 0.05$) in the PSA than in the SA mice.

The body weights recorded at various stages of the experiment showed that at no time during the study was there any statistically significant difference ($P < 0.05$ by Student's t test) between the weights of the animals in the two groups. Initial and terminal body weights were, in males, 26 and 43 g in the SA group and 25 and 42 g in the PSA group and, in females, 19 and 30 g and 20 and 34 g in the SA and PSA groups, respectively. Similarly there was no evidence of any effect of PSA treatment on the organ weights or relative organ weights (Table 2) of animals surviving to wk 80 or on the results of the haematological analyses conducted at wk 12, 26, 51 and 80 ($P > 0.05$ by Student's t test).

The lesions identified on histopathological examination, listed in Table 3, were those normally found in ageing mice. There were increases in the incidences of fatty degeneration of the liver and chronic inflammation of the kidney in the PSA male mice, but these differences were not statistically significant. In addition, the incidence of chronic renal inflammation was less in the PSA females than in the SA group. The incidences of all other lesions were similar in both treatment groups within each sex. The tumours (Table 4) included benign tumours of the lungs, ovaries, mammary tissue, uterus and subcutaneous tissue. Without exception these tumours were present with a similar incidence in animals of both treatments or were confined to the SA mice. Malignant tumours of the reticulo-endothelial system were found in one male SA mouse, in three male PSA mice and in two PSA females. A single anaplastic carcinoma of the anus was found in a PSA female mouse.

Table 1. *Cumulative mortality in mice fed diets containing 1·2% sorbic acid or 1·2% sorbic acid contaminated with 1000 ppm parasorbic acid for 80 wk*

Wk of treatment	Total no. of animals dead			
	Males		Females	
	SA	PSA	SA	PSA
20	0	0	0	1
40	1	0	0	2
60	1	2	0	5
70	1	5	4	7
72	1	5	4	10
76	4	5	4	12
80	5	6	5	16*

SA = Group fed diet containing 1·2% sorbic acid
PSA = Group fed diet containing 1·2% sorbic acid contaminated with 1000 ppm parasorbic acid
The figures represent the total number of rats dead from groups of 48: those marked with an asterisk differ significantly ($P < 0.05$ by chi-square test) from those of the SA group.

DISCUSSION

The slight increase in deaths in the female PSA mice compared with the SA group, although statistically significant during the last 3 wk of the experiment, was probably not related to treatment. The five animals dead before wk 60 were killed because of ill-health. In three of these animals the lesions responsible for their moribund condition were severe middle-ear infection, generalized lymphoblastoma and papillary adenoma of the lungs. In a fourth the only pathological finding was cloudy swelling of the liver. All of these lesions are common in mice and there is no evidence to implicate parasorbic acid in their formation. The remaining interim deaths in this group were of differing aetiology. In addition there was an unusually low mortality rate in the SA females compared with other studies in our laboratories. Grasso, Hardy, Gaunt, Mason & Lloyd (1974) found a death rate of 50% in untreated females of this strain, while Hendy *et al.* (1976) reported a 31% death rate in control females and 42% in females given 1% sorbic acid in their diet. Hence the rate in the PSA group

Table 2. *Relative organ weights of mice fed diets containing 1·2% sorbic acid or 1·2% sorbic acid contaminated with 1000 ppm parasorbic acid*

Sex and group	No. of mice examined	Body weight (g)	Relative organ weight (g/100 g body weight)						
			Brain	Heart	Liver	Spleen	Kidney	Stomach	Small intestine
Male									
SA	43	38	1·21	0·67	4·67	0·31	1·90	1·13	5·17
PSA	42	38	1·22	0·60	4·87	0·33	1·83	1·18	5·20
Female									
SA	43	29	1·57	0·61	5·01	0·50	1·43	1·45	5·78
PSA	32	30	1·57	0·58	5·20	0·52	1·47	1·50	5·82

SA = Group fed diet containing 1·2% sorbic acid
PSA = Group fed diet containing 1·2% sorbic acid contaminated with 1000 ppm parasorbic acid
The figures are means for the numbers of mice shown; the differences between the two groups were not statistically significant ($P > 0.05$ by Student's t test).

Table 3. *Incidence of histopathological lesions in mice fed diets containing 1·2% sorbic acid or 1·2% sorbic acid contaminated with 1000 ppm parasorbic acid*

Organ and histopathological finding	Incidence of lesion in			
	Male		Female	
	SA	PSA	SA	PSA
No. of mice examined ...	47	48	47	44
Lung				
Peribronchial lymphocytes	0	1	0	1
Chronic inflammation	0	1	0	1
Congestion	2	2	0	0
Metaplastic cells	1	0	0	1
Liver				
Deposits of lymphocytes	0	0	0	1
Perisinusoidal lymphocytes	0	0	3	0
Cloudy swelling	0	0	2	4
Vacuoles	1	1	1	1
Fatty degeneration	2	8	1	3
Nodular hyperplasia	8	7	5	3
Brown macrophages	1	0	4	0
Lymphoid hyperplasia	0	0	1	0
Kidney				
Chronic inflammation	6	13	13	4
Degenerative changes	5	2	6	5
Lymphoid hyperplasia	1	1	0	0
Deposits of lymphocytes	0	1	0	0
Perivascular lymphocytes	5	2	15	4
Spleen				
Lymphoid hyperplasia	1	1	2	0
Ovaries				
Follicular cysts	—	—	3	3
Uterus				
Cystic hyperplasia	—	—	3	0
Testis				
Atrophy	2	2	—	—
Ileum				
Lymphoid hyperplasia	1	0	0	0
Skin				
Chronic inflammation	2	0	1	0
Lymph nodes				
Hyperplasia	2	2	0	0

SA = Group fed diet containing 1·2% sorbic acid

PSA = Group fed diet containing 1·2% sorbic acid contaminated with 1000 ppm parasorbic acid

The figures represent the incidence of the finding in the numbers of mice shown; none differed significantly ($P > 0·05$ by chi-square test) from the incidence of the same finding in the SA group.

in the present study was within that which has been experienced with the strain of mice used. It was concluded that the increased number of deaths in this group was a chance occurrence unrelated to treatment.

Increases in the incidence of fatty degeneration of the liver and of chronic renal inflammation were found in male mice fed parasorbic acid. The estimated probability of these occurrences was $>0·05$ (chi-square test). Moreover these are common findings in mice and incidences similar to those in the PSA animals have been reported in untreated mice (Grasso *et al.* 1974) suggesting that these differences represented variations within the normal range rather than any effect of parasorbic acid. This lack of treatment-related effect is supported by the absence of any similar findings in the female mice given the same treatment. Indeed, in the case of the renal inflammation in females, there was a difference in the opposite direction, namely a lower incidence in the PSA group. These considerations show that the differences in the incidence of these lesions between the two groups are of no significance in the toxicological evaluation of parasorbic acid.

Without exception, all the benign tumours found during the study were those commonly encountered in untreated mice (Cloudman, 1956) and they either occurred only in the SA mice or were found in a

Table 4. *Incidence of tumours in mice fed diets containing 1·2% sorbic acid or 1·2% sorbic acid contaminated with 1000 ppm parasorbic acid*

		Incidence of tumour in			
		Male		Female	
		SA	PSA	SA	PSA
Organ and tumour	No. of mice examined ...	47	48	47	44
Lungs					
Adenoma		13	16	9	8
Liver					
Hepatoma		1	0	1	1
Ovaries					
Granuloma-cell tumour		—	—	1	0
Luteoma		—	—	1	0
Mammary tissue					
Fibroadenoma		—	—	1	0
Uterus					
Fibroma		—	—	1	0
Subcutaneous tissue					
Fibroma		2	0	1	1
Reticulo-endothelial system					
Lymphoblastoma		1	3	0	2
Anus					
Anaplastic carcinoma		0	0	0	1

SA = Group fed diet containing 1·2% sorbic acid
PSA = Group fed diet containing 1·2% sorbic acid contaminated with 1000 ppm parasorbic acid
The figures represent the numbers of mice affected among the numbers shown.

similar incidence in both groups. Two generalized lymphoblastomas and one anaplastic carcinoma of the anus were found in three female PSA mice. Lymphoblastoma was also found in one male SA animal. This is a relatively common tumour in untreated mice and has been found on previous occasions in these laboratories (Mason, Gaunt, Butterworth, Hardy, Kiss & Grasso, 1974). Similarly, malignant epithelial tumours of the anal region, although less common, have been observed in previous studies in this strain of mice (P. Grasso, unpublished observations 1974). Hence it seems that neither of these tumours can be taken as representing a carcinogenic effect of parasorbic acid.

There is no evidence from this lifespan study in mice to indicate that sorbic acid contaminated with 1000 ppm parasorbic acid has any carcinogenic effect. Diet containing 1·2% sorbic acid contaminated with 1000 ppm parasorbic acid represents an intake of approximately 1·7 mg parasorbic acid/kg. The acceptable daily intake of sorbic acid suggested in the report of the Joint FAO/WHO Expert Committee on Food Additives (1967) was 12·5 mg/kg. Contamination of this with 1000 ppm parasorbic acid provides an intake of the latter of 12·5 μg/kg. There has been no systematic study of the parasorbic acid content of commercially available sorbic acid nor of the extent to which parasorbic acid is formed during the preparation of foods, but Murphy & Wardleworth (1973) were unable to detect its presence in any of the sorbic acid samples at their disposal. In addition, the Ministry of Technology: Laboratory of the Government Chemist (1970) stated that parasorbic acid was unlikely to occur as a by-product of the manufacturing process for sorbic acid and, equally, that the conversion of sorbic to parasorbic acid was unlikely under the conditions of food processing or cooking. It seems improbable, therefore, that the human intake of this material could ever approach the levels used in the studies described here and consequently the hazard must be considered negligible.

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PHARMACOKINETIC PROFILE OF DIAMIDFOS IN RATS

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Abstract—Clearance of ^{14}C activity from plasma and elimination of ^{14}C from the body of rats were determined after administration of [^{14}C]Diamidfos in single iv doses of 1 and 25 mg/kg. Clearance of ^{14}C activity from plasma was by an apparent first order process with half-life values of 2·94 and 3·08 hr, respectively. Urinary elimination of ^{14}C activity from the body was biphasic at both dose levels. The half-life values for the rapid and slow phases of elimination were 3·08–3·47 and 10–11 hr, respectively, at both dose levels. Approximately 90% of administered ^{14}C activity was excreted in the urine and more than 95% of this was excreted during the first 36 hr. The ^{14}C activity eliminated in the urine consisted of three ^{14}C-labelled metabolites and [^{14}C]Diamidfos. Each metabolite and Diamidfos accounted for approximately 25% of the ^{14}C activity in the urine. The metabolites were identified by gas chromatography–mass spectrometry, infra-red spectroscopy and nuclear magnetic resonance spectrometry. The metabolites were *N*-methylphosphorodiamidate, *N*-formyl-*N'*-methylphosphorodiamidate and phenol. Approximately 7% of administered ^{14}C activity was eliminated in the faeces. The only tissue with detectable amounts of ^{14}C activity was the liver, 72 hr after dosing. Overall recovery of ^{14}C activity was 97% of the calculated dose. Clearance of ^{14}C activity from plasma and elimination of ^{14}C from the body were determined also after a single oral dose of [^{14}C]Diamidfos at 25 mg/kg. The time profiles of plasma ^{14}C activity after administration of [^{14}C]Diamidfos at 25 mg/kg both iv and orally were directly superimposable. In addition, 87% of the ^{14}C activity was found in urine after oral administration. It appears, therefore, that orally administered [^{14}C]Diamidfos was extensively, if not completely, absorbed. There was no evidence of non-linear pharmacokinetics and no indication that significant amounts of Diamidfos and/or its metabolites would be retained in the body over long periods of time or would accumulate on repeated administration.

INTRODUCTION

Diamidfos (phenyl *N,N'*-dimethylphosphorodiamidate; C_6H_5—O—(O)P(NH—CH_3)—NH—CH_3) is a nematocide currently used against soil-born nematodes on tobacco and of potential use on cotton and in food crops. Data from this laboratory have shown that the acute oral LD_{50} of Diamidfos is 140 and 200 mg/kg in male and female rats, respectively, and that diets incorporating 10 and 30 ppm Diamidfos were tolerated by rats for 90 days without any adverse effects. Depressed plasma, red cell and brain cholinesterases were noted with a dietary level of 100 ppm.

Since data on the metabolism and pharmacokinetic profile of Diamidfos in laboratory animals were not available but would aid the interpretation of the results of toxicity studies in animals, a study was undertaken in rats to determine, after iv administration of [^{14}C]Diamidfos at 1 and 25 mg/kg, the rate of clearance of ^{14}C activity from plasma, the rate of excretion and amount of ^{14}C activity and Diamidfos in urine, the amount of ^{14}C activity eliminated in faeces, the concentration of ^{14}C activity in selected tissues at autopsy, and the number of urinary metabolites and their relative concentration in the urine, as well as to achieve a preliminary structural identification of the major urinary metabolites. The rate and extent of absorption and clearance of ^{14}C activity following oral administration of [^{14}C]Diamidfos were also determined.

EXPERIMENTAL

Animals and treatment. Male and female Sprague–Dawley (Spartan substrain) rats weighing approximately 200 g were used in the study. Animals were given free access to food and water throughout the experiment and were acclimatized to their environment for 3 days prior to the administration of Diamidfos. They were housed separately in stainless-steel metabolism cages designed for the separate collection of urine and faeces. The rats were maintained in an air-conditioned room (24 ± 1°C) with a 12-hr light–dark cycle. Jugular cannulas were surgically implanted in rats under methoxyfluorane anaesthesia (Harms & Ojeda, 1974) 72 hr before administration of Diamidfos.

All animals were dosed between 8.00 and 9.00 hr. Rats used in the excretion studies were returned to their cages and not removed until killed. Rats used in the plasma studies were removed from their cages only for the taking of blood samples from the jugular cannula. Samples were taken at 10 and 30 min and at 1, 2, 3, 4, 6, 8, 12, 18 and 24 hr after administration of [^{14}C]Diamidfos at 1 and 25 mg/kg, and additionally at 30 and 36 hr after administration at 25 mg/kg. Samples were collected in a heparinized capillary tube, sealed at one end, and centrifuged for 5 min in a microhaematocrit centrifuge. The haematocrit tube was broken near the plasma–red cell interface and the plasma was drained into pre-weighed scintillation vials for ^{14}C analysis.

Urine traps were immersed in dry-ice baths throughout each 12-hr collection interval. Samples of urine and faeces were stored at −2°C until analysed for ^{14}C activity and/or Diamidfos.

Rats were killed by decapitation at the termination of the excretion studies, 72 hr after dosing. Liver, kidney, a sample of perirenal fat, skeletal muscle tissue

and brain were removed and the carcass was skinned. All tissues were stored in tared containers at −2°C. Each metabolism cage was washed with water and the cage wash was analysed for ^{14}C activity.

Test material. Uniformly ring-labelled [^{14}C]Diamidfos (specific activity 2·15 μCi/mg) and unlabelled Diamidfos (Dow Analytical Standard AGR No. 23, 612B) were used. The radiochemical purity of [^{14}C]Diamidfos was established as 98·6% by two-dimensional thin-layer chromatography (TLC) using the following systems: Silica gel (Brinkmann, 0·25 mm thick) with methanol–acetone, 1:19 (R_F 0·41), silica gel (Brinkman, 0·25 mm thick) with glacial acetic acid–acetone, 1:99 (R_F 0·58) or alumina (Brinkmann, 0·25 mm thick) with methanol–acetone, 1:4 (R_F 0·52).

Administration. Aliquots of labelled and unlabelled Diamidfos were dissolved together in physiological saline and were administered to rats by the intravenous jugular cannula. Groups of four rats (two male, two female) received either 1 or 25 mg/kg in a volume of 0·5 ml. The dosing solutions were prepared to contain 10 μCi/ml at both dose levels (i.e. 25 μCi/kg body weight). A further group received 25 mg/kg orally from a syringe fitted with a feeding needle.

Estimation of ^{14}C activity. All ^{14}C activity was measured by liquid scintillation counting on a Nuclear Chicago Mark II Liquid Scintillation Counter using external standard ratios to determine counting efficiency. The counting efficiencies were checked periodically with an internal standard of [^{14}C]toluene. Urine, plasma and cage wash were counted after addition of water and Aquasol. Faeces, carcass, liver, kidney and brain were prepared as 33% aqueous homogenates. A portion of the homogenate was oxidized in a Beckman Biological Material Oxidizer. Perirenal fat and skeletal muscle were oxidized directly. Carbon dioxide formed during the oxidation was trapped in 8 ml of a solution of 5 M-2-aminoethanol in 2-methoxyethanol. ^{14}C activity was quantified after addition of 10 ml of a scintillation mixture containing 4 g PPO and 0·1 g POPOP/litre of toluene–2-methoxyethanol (1:1, v/v). Recovery of ^{14}C activity from oxidized samples spiked with [^{14}C]ATP was 95 ± 5%.

Quantification of Diamidfos metabolites. TLC was used to separate urinary fractions containing ^{14}C activity. To determine the number of urinary metabolites after iv administration, 100-μl aliquots of the urine collected at 12, 24 and 36 hr after injection of Diamidfos at each dose level were spotted on channelled silica-gel (0·25 mm thick, Brinkmann) plates. The vertical channels were made by scoring the absorbant with a scalpel blade at 10-mm horizontal intervals. Standard [^{14}C]Diamidfos was chromatographed with the urine samples. The thin-layer plates were developed three times with methanol to the top edge of the spotted urine, then developed in glacial acetic acid–acetone (1:99) to the top of the plate. The plates were scanned and areas on the plate containing ^{14}C activity were marked and scraped. The absorbant was transferred to scintillation vials and counted for ^{14}C activity.

Isolation of urinary metabolites. TLC and mass spectrometry were used to identify the structures of the urinary metabolites. Urine from the 12-hr collection was streaked across a silica-gel thin-layer (0·25 mm) plate, 15 mm from the bottom, and the plate was developed twice with acetone to the top of the plate. The plate was scanned and areas containing ^{14}C activity were marked. The silica gel on the TLC plate between the areas of radioactivity was removed. The areas containing ^{14}C activity were tapered to a point at one end and flared at the other end (See Fig. 1). The plate was then developed in the second dimension with methanol for 24 hr in a tank with the cover plate left slightly open to allow continuous development. The plate was scanned a second time. All ^{14}C activity was localized in the tapered point of each channel.

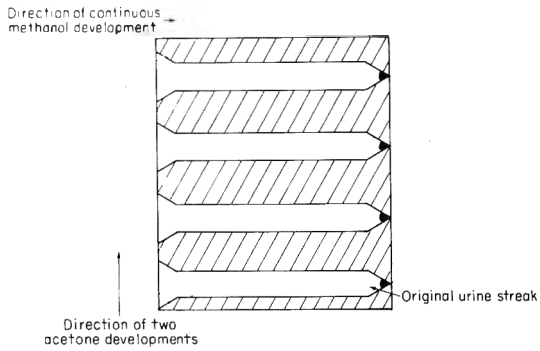


Fig. 1. Thin-layer chromatography system for isolation of urinary metabolites for mass-spectral and infra-red examinations: □, silica gel; ▨, silica gel removed from plate after two acetone developments; ◨, final location of ^{14}C-labelled urine metabolites.

Mass spectrometric analysis. The silica gel from the tip of each channel was removed from the TLC plate and placed in a separate capillary tube. The capillary tubes were inserted into the direct probe inlet of a Finnigan Model M-3000 Quadrupole Mass Spectrometer. The instrument was operated with a source temperature of 40°C, a probe temperature programmed from 40 to 400°C and an ion energy of 70 eV. The mass range of 45–500 *m/e* was scanned at a rate of 30 scans/min. The mass spectrum of each metabolite was recorded and examined for salient structural characteristics. Both electron impact (EI) and chemical ionization (CI) mass spectra were obtained, the latter by using isobutane as the ionizing gas.

Infra-red spectrophotometric analysis. Additional samples of the metabolites were extracted from the silica gel with methylene chloride and evaporated to dryness separately on small amounts of potassium bromide. Micropellets were pressed from the resulting mixture and centered in a Perkin-Elmer 6× beam condenser mounted in the sample compartment of an FTS-14 spectrometer to obtain the infra-red (IR) spectrum.

Nuclear magnetic resonance spectrometry. An additional methylene chloride extract was evaporated, and the residues were dissolved separately in deuterated chloroform. The 100 MHz proton nuclear magnetic resonance (NMR) spectra of the C^2HCl_3 solutions were obtained on a Varian XL-100 spectrometer by the PFT method with overnight time-averaging to produce usable signals.

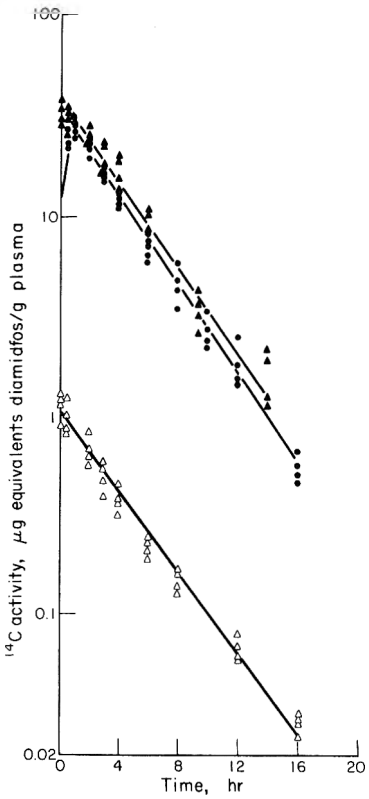


Fig. 2. The concentration of ^{14}C activity expressed as μg equivalents Diamidfos/g plasma as a function of time following iv administration of [^{14}C]Diamidfos at 1 (△) and 25 mg/kg (▲) and an oral administration at 25 mg/kg (●).

RESULTS

The concentration of ^{14}C activity in the plasma of treated rats is shown in Fig. 2 as a function of time. Clearance of ^{14}C activity followed apparent first order kinetics, the semilogarithmic plot of concentration versus time giving a straight line. The rate of clearance of ^{14}C activity from plasma and the volume of distribution at the low and high doses are presented in Table 1. The rate constants and half-life values were calculated by linear regression analysis of the logarithmically transformed data.

Table 2 shows the total excretion of ^{14}C activity in the urine and faeces throughout the study expressed as a percentage of the dose. The urinary excretion during successive 12-hr intervals after iv administration of [^{14}C]Diamidfos is shown in Fig. 3 where the percentage of dose excreted is plotted versus time on semilogarithmic co-ordinates.

The elimination of ^{14}C activity in the urine at both dose levels after iv administration appeared to be biphasic. The rapid excretion phase of ^{14}C activity persisted for 36 hr after administration of the dose and less than 1% of the administered dose was eliminated between 48 and 72 hr. The rate constants and half-life values for both phases of excretion were calculated by a feathering technique and are presented in Table 3.

The only tissue, including carcass, with a measurable quantity of ^{14}C activity was the liver, in which 0·10 and 0·18% of the administered dose remained 72 hr after iv injection of [^{14}C]Diamidfos at 1 and 25 mg/kg respectively.

To assess the overall recovery, the cage wash was counted for ^{14}C activity, and the results are presented in Table 2.

After separation of the urinary metabolites by TLC, four areas on the plate contained significant quantities of ^{14}C activity. Approximately 25% of the ^{14}C activity was associated with material with the same R_F value as Diamidfos, approximately 25% remained at the origin (metabolite A), and two additional areas, each containing approximately 25% of the ^{14}C activity, were located at R_F values greater than Diamidfos (metabolites B and C). The percentage of ^{14}C activity on the thin-layer plate associated with Diamidfos and its metabolites at various times is shown in Table 4.

The CI and EI mass spectra of metabolite A are shown in Fig. 4a,b. The CI spectrum indicates that the molecular weight of metabolite A is 186. Enzymatic *N*-demethylation in the liver is well documented, and *N*-demethylation of Diamidfos would result in a molecule of molecular weight 186 (C_6H_5—O—(O)P(NH—CH_3)—NH_2).

The EI spectrum supports this structure in several ways. First the base peak of the spectrum is *m/e* 93 which corresponds to two fragments associated with the cleavage of the phenoxy to phosphorus bond (Quayle, 1959), [C_6H_5—O]$^+$ and [(O)P(NH_2)—NH—CH_3]$^+$. Secondly the loss of H_2O from the molecular ion (Budzikiewicz, Djerassi & Williams, 1967) would give the peak at *m/e* 168 [C_6H_5—O—P(=N—CH_3)=NH]$^{\cdot+}$. Thirdly, the loss of an NH—CH_3 radical would give the even electron ion at *m/e* 156, [C_6H_5—O—P(O)—NH_2]$^+$. Cleavage

Table 1. *Rate constants* (k) *and half-life values* ($t_{1/2}$) *for the clearance of* ^{14}*C activity from plasma and the volumes of distribution* (V_d) *following a single dose of* [^{14}*C*]*Diamidfos*

Dose (mg/kg)	Route of administration	k* (hr)	$t_{1/2}$ (hr)	V_d† (ml/kg)
1	iv	0·225 ± 0·012	3·08 ± 0·16	802 ± 85
25	iv	0·236 ± 0·023	2·94 ± 0·29	694 ± 103
25	oral	0·229 ± 0·020	3·02 ± 0·30	NC

NC = Not calculated

*Calculated by linear regression analysis.

†Calculated by dividing the dose (mg/kg) by the plasma concentration of Diamidfos prior to excretion, a value derived by projection of plasma-clearance curves back to 0 hr.

Values are means ± SD for two male and two female rats.

Table 2. *Recovery of ^{14}C activity in urine, faeces, tissues and cage wash in the 72-hr period after administration of [^{14}C]Diamidfos*

Dose (mg/kg)	Route	^{14}C activity (% of dose) in Urine	Faeces	Liver*	Cage wash	Total
1	iv	89·63 ± 4·75	7·00 ± 1·23	0·10 ± 0·03	0·94 ± 0·28	97·67 ± 5·42
25	iv	87·83 ± 2·78	7·01 ± 2·98	0·18 ± 0·04	1·46 ± 0·80	96·47 ± 2·95
25	oral	87·15 ± 1·94	7·96 ± 2·13	0·11 ± 0·04	0·70 ± 0·21	95·92 ± 2·73

*No activity was detected in any other tissues, including the carcass.
Values are means ±SD for two male and two female rats.

of the same N—P bond could also result in the formation of the $H_2N{=}CH_2^+$ ion (*m/e* 30) depending on the charge location.

The IR data support the proposed structure. The IR spectrum contains bands at 1598, 1497, 1076, 1020 and 1011 cm^{-1} from in-plane vibrations of the phenyl group, and bands at 775 and 696 cm^{-1} from out-of-plane vibrations of the phenyl group. Thus, the benzene ring is monosubstituted. A band at 923 cm^{-1} is characteristic of the C_6H_5—O—P group (O—P stretching) and a strong band at 1220 cm^{-1} results from P=O stretching. Bands at 2938 and 2828 cm^{-1} support the presence of an N—CH_3 group and bands at 1120 and 825 cm^{-1} support the presence of a P—N—CH_3 group. Broad bands near 1400 cm^{-1} could result from P—(NH) bending and a band at 1574 cm^{-1} from P—(NH_2) bending.

The CI and EI mass spectra of the component with the same R_F value as Diamidfos are shown in Fig. 5a,b. These spectra are identical with the reference spectra of Diamidfos and confirm the identity of [^{14}C]Diamidfos.

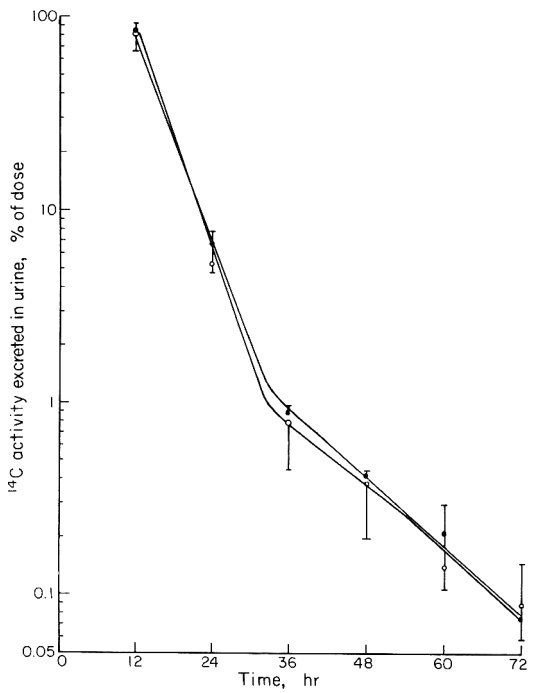
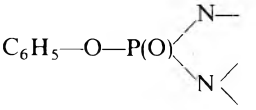


Fig. 3. ^{14}C activity excreted in urine during successive 12-hr intervals following iv administration of [^{14}C]Diamidfos at 1 (●) and 25 (○) mg/kg. Each point represents the mean ±SD for two male and two female rats.

The CI and EI mass spectra of metabolite B are shown in Fig. 6a,b. The CI spectrum shows that the molecular weight of metabolite B was 214 and not 187 as indicated by the EI spectrum and confirms the instability of the molecular ion already noted in the EI spectrum. One possible structure of metabolite B could arise from enzymatic *N*-methylation of Diamidfos to give C_6H_5—O—(O)P(NH—CH_3)—N(CH_3)$_2$. This compound was synthesized and the mass spectrum proved that it was not metabolite B.

Secondary amines are dealkylated by microsomal mono-oxygenases to primary amines and aldehydes and, in certain cases, the *N*-hydroxylalkyl intermediates are stable. Diamidfos would give [C_6H_5—O—(O)P(NH—CH_3)—NH—CHO ↔ C_6H_5—O—(O)P(NH—CH_3)—N=CHOH]. These structures fit two of the criteria imposed by the mass spectra: the instability of the molecular ion and the ability to rearrange with loss of HCN to yield [C_6H_5—O—(O)P(NH—CH_3)—OH]$^{\cdot+}$.

The IR spectrum supports the proposed structure. This sample has bands at 1597, 1494, 1460, 1076, 1029 and 1010 cm^{-1}, resulting from in-plane vibrations of a phenyl group and bands at 772 and 694 cm^{-1} from out-of-plane vibrations of a phenyl group. These data show conclusively that the compound contains a mono-substituted phenyl group. Medium intense bands are observed at 1200 and 947 cm^{-1}, and a weak–medium band is observed at 1167 cm^{-1}. These bands are assigned as P=O stretching (1200 cm^{-1}), (C_6H_5—O)P stretching (1167 cm^{-1}) and C_6H_5(O—P) stretching (947 cm^{-1}). These facts indicate that the compound contains a C_6H_5—O—P(O)= group. In addition, with the P=O stretching vibrations occurring at 1200 cm^{-1}, it is certain that the other two substituents on the phosphorus atom are nitrogen, otherwise the P=O stretching vibration would occur at a higher frequency. Hence, the compound contains the group



with hydrogen on at least one of the nitrogen atoms. A band near 1115 cm^{-1} could result from C—N—P stretching for the P—NH—CH_3 group.

The absorption of the NH stretching vibration has its maximum near 3250 cm^{-1}, and the band is broad. It is not possible from this to determine whether the molecule contains P—NH_2 and/or P—NH groups. The spectrum does not show a band *c.* 1570 cm^{-1}; therefore, the sample does not contain a P(=O)NH_2

Table 3. *Rate constants* (k) *and half-life values* ($t_{1/2}$) *for the urinary excretion of* ^{14}C *activity from rats after iv administration of* [^{14}C]*Diamidfos*

Dose (mg/kg)	Excretion phase	k (hr^{-1})	$t_{1/2}$ (hr)
1	α	0·200 ± 0·054	3·47 ± 0·74
	β	0·069 ± 0·033	10·0 ± 3·2
25	α	0·224 ± 0·045	3·09 ± 0·51
	β	0·063 ± 0·019	11·0 ± 2·5

Values are means ± SD for two male and two female rats.

group. Broad absorption near 1400 cm^{-1} could result from NH bending of the P(=O)NH group.

The C=O frequency is unusually high for an amide group such as CH_3—C(O)—NH— or HC(O)—NH—. However, if we consider that the N—H group is hydrogen-bonded with P=O rather than C=O, and that the P=O inductive effect upon C=O would be high, the 1702 cm^{-1} frequency assignment for the C=O stretching vibration for the H—C(O)—NH—P(O)= group is not unreasonable.

The NMR spectrum of metabolite B contains features that support a P—NH—CH_3 assignment, i.e. CH_3 as a 13 Hz doublet of 6 Hz doublets at a 2·87 ppm shift from TMS. The HC(O)—N assignment is supported, but less conclusively, by a single broad absorption at 8·52 ppm. The NH proton absorptions are not discernible, presumably because they are broadened by nitrogen splitting and become lost in baseline noise.

Metabolite C had the same R_F value as phenol in three different TLC systems. The EI mass spectrum of metabolite C shown in Fig. 7 is not identical to that of phenol, but, when viewed in the light of the TLC data, the spectral character is close enough to assign this compound the phenol structure, C_6H_5OH.

DISCUSSION

After an iv dose of [^{14}C]Diamidfos at 1 or 25 mg/kg, ^{14}C activity was cleared from rat plasma by an apparent first order process, the rate constants being 0·225/hr ($t_{1/2}$, 3·08 hr) and 0·236/hr ($t_{1/2}$, 2·94 hr), respectively. There were no statistical differences between the rates of clearance at the two dose levels (t test, $P > 0·05$). Therefore, clearance of ^{14}C activity from plasma is not dose dependent within the range of dose levels studied. In addition, statistical analysis (paired t test) of the logarithmically transformed data revealed that the plasma concentration of ^{14}C activity was not sex dependent.

Examination of the ^{14}C activity in the plasma in relation to time after oral administration of [^{14}C]Diamidfos at 25 mg/kg revealed rapid absorption and clearance of ^{14}C activity from the plasma. Peak plasma levels were obtained approximately 30 min after administration of the dose and the rate constants for clearance from the plasma were essentially identical to those calculated after iv administration. The ^{14}C activity of the plasma in relation to time was the same after iv and oral administration

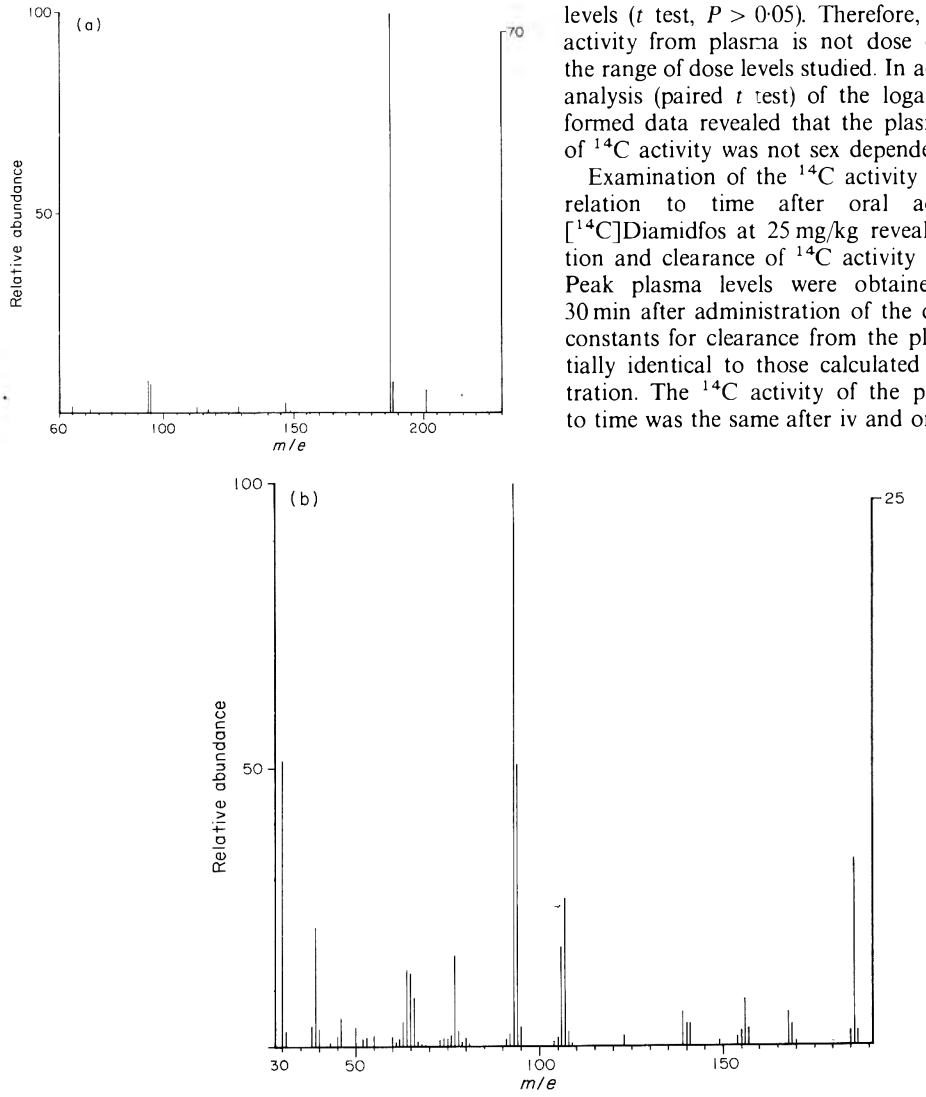


Fig. 4. Chemical ionization (a) and electron impact (b) mass spectra of metabolite A.

Table 4. *^{14}C activity associated with [^{14}C]Diamidfos and its metabolites A, B and C in urine collected from rats after iv administration of [^{14}C]Diamidfos*

Dose (mg/kg)	Time (hr)	Radioactivity (% of administered dose) represented by Metabolite A (R_F 0·13)	Diamidfos (R_F 0·46)	Metabolite B (R_F 0·55)	Metabolite C (R_F 0·61)
1	12	23 ± 7	32 ± 9	28 ± 7	17 ± 6
	24	25 ± 6	23 ± 8	28 ± 6	24 ± 7
	36	27 ± 4	18 ± 3	22 ± 6	33 ± 6
25	12	33 ± 7	23 ± 2	26 ± 6	18 ± 7
	24	33 ± 7	21 ± 4	23 ± 4	23 ± 9
	36	31 ± 10	21 ± 8	24 ± 3	24 ± 10

Values are means ±SD for three determinations on the urines of two male and two female rats.

of a dose of 25 mg/kg indicating that orally-administered ^{14}C activity was extensively, if not completely, absorbed. This point is reinforced by the finding of approximately 87% of ^{14}C activity in the urine following oral dosing with Daimidfos.

Projection back to the *y*-axis (time = 0) of the plasma clearance curves after iv dosing allows calculation of the concentration of Diamidfos in plasma prior to excretion. This concentration is dependent on the distribution of Diamidfos to tissues and its subsequent entry into cells and/or tissue spaces. The volume of distribution calculated by dividing the dose (mg/kg) by the concentration of Diamidfos at 0 hr is not significantly different at the two dose levels.

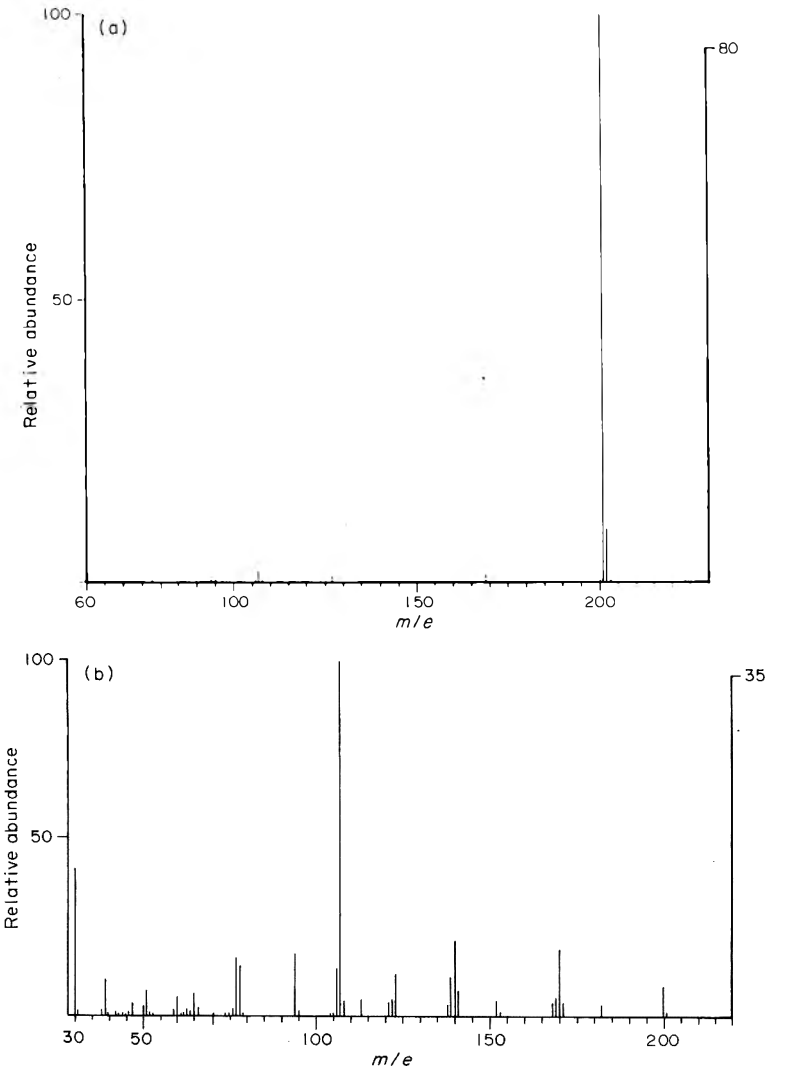


Fig. 5. Chemical ionization (a) and electron impact (b) mass spectra of Diamidfos isolated from urine.

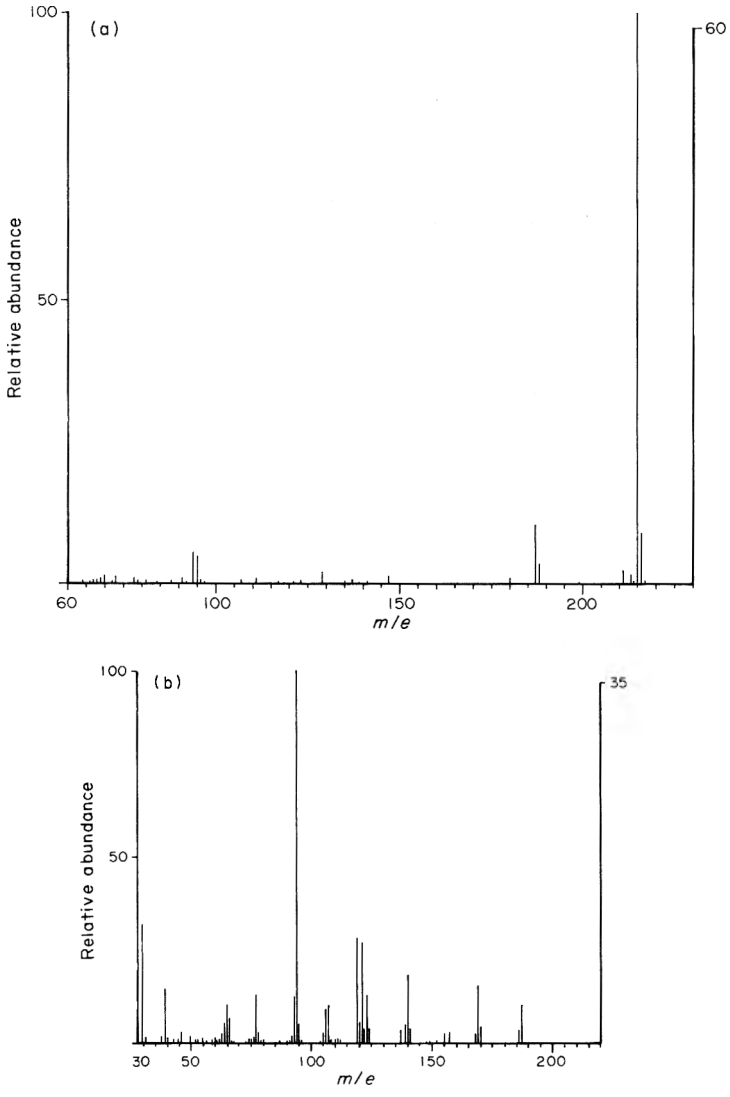


Fig. 6. Chemical ionization (a) and electron impact (b) mass spectra of metabolite B.

This indicates that at a high dose Diamidfos does not enter tissues or organs that are not exposed to Diamidfos at a low dose. The concentration of Diamidfos in tissues and/or organs should be proportional to the dose. The excretion of ^{14}C activity in the urine accounts for approximately 93% of the ^{14}C activity administered iv as [^{14}C]Diamidfos at 1 and 25 mg/kg. Approximately 7% of the ^{14}C activity is eliminated by biliary excretion and subsequent removal from the body in the faeces. The excretion of ^{14}C activity from the body in the urine is biphasic and essentially superimposable when plotted as the percentage of ^{14}C activity eliminated versus time at the two iv dose levels. The rate constants for the excretion of ^{14}C activity in the urine at both dose levels are not statistically different. More than 95% of the ^{14}C activity excreted in the urine was eliminated during the first 36 hr ($t_{1/2}$ 3–3·5 hr) after administration of the dose. Less than 2% of the administered dose was eliminated between 48 and 72 hr at both dose levels, the half-life being 10–11 hr. There is no indication that significant amounts of ^{14}C activity (Diamidfos and/or ^{14}C-labelled metabolites) would be retained in the body for long periods or that it would accumulate on repeated administration. The only tissue with detectable levels of ^{14}C activity 72 hr after iv doses of [^{14}C]Diamidfos at 1 and 25 mg/kg was the liver, which contained between 0·10 and 0·18% of the

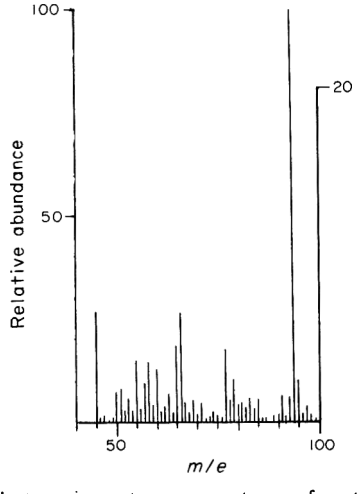


Fig. 7. Electron impact mass spectrum of metabolite C.

administered ^{14}C activity at this time. ^{14}C activity was not detected in the carcass.

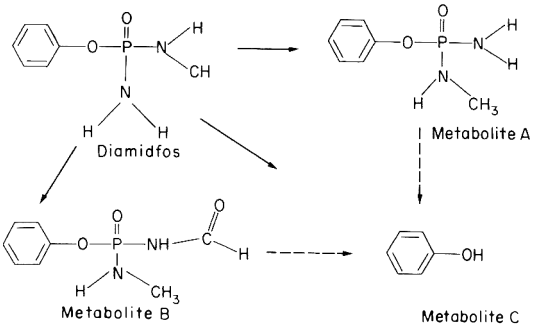


Fig. 8. Proposed metabolic pathways for Diamidfos.

Conclusion

Diamidfos is metabolized, probably in the liver, and eliminated by the kidney as three major metabolites plus the parent compound. Diamidfos and the three metabolites each account for approximately 25% of the administered ^{14}C activity. The metabolites were identified using gas chomatography–mass spectrometry, infra-red spectroscopy and nuclear magnetic resonance spectrometry. The metabolites were *N*-methylphosphorodiamidate, *N*-formyl-*N'*-methylphosphorodiamidate and phenol.

A scheme for the metabolism of Diamidfos is proposed on the basis of the results of these investigations (Fig. 8).

As shown in Fig. 8, Diamidfos is converted to metabolites A, B and C. It cannot be determined from the data in this study whether A and B are converted to C. The fraction of metabolites excreted in the urine remains relatively constant over the first three 12-hr excretion intervals, indicating that the rate of metabolism of Diamidfos is first order. In addition, the proportion of each metabolite was unaffected by dose levels suggesting an absence of dose-dependent saturation phenomena.

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SHORT-TERM PERORAL TOXICITY OF ETHYLIDENE GYROMITRIN IN RABBITS AND CHICKENS

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Abstract—Ethylidene gyromitrin, the main toxic compound in false morel (*Gyromitra esculenta*), was given in drinking-water to groups of five rabbits and ten chickens of each sex in doses of 0 (control), 0·05, 0·5 or 5·0 mg/kg/day for 90 days. The following tests and measurements were made during this period or at autopsy: weight increase, relative organ weights, haematological measurements, serum and urine analyses, including the determination of ethylidene gyromitrin in the urine, and macro- and histopathological examination. The no-effect level of ethylidene gyromitrin was estimated as 0·5 mg/kg/day for rabbits and 0·05 mg/kg/day for chickens. The most important biological effects in rabbits were degenerative changes in the tubular cells of the kidneys, while in chickens defects were also detected in the heart muscle and liver parenchymal cells.

INTRODUCTION

False morel, *Gyromitra esculenta* (Pers.) Fr., is a wild mushroom commonly found in areas from Central Europe to the north of the Arctic Circle and also known in North America. In spite of its toxicity when fresh, home or industrially prepared *G. esculenta* is eaten cooked or dried in many countries and is considered to be a delicacy.

The toxic compounds in fresh *G. esculenta* are thought to be hydrazones, which are rarely found in nature. The main toxic compound is acetaldehyde *N*-methyl-*N*-formylhydrazone, known as ethylidene gyromitrin or gyromitrin (List & Luft, 1967). *G. esculenta* also contains homologues of ethylidene gyromitrin, such as the pentanal, 3-methylbutanal and hexanal *N*-methyl-*N*-formylhydrazones (Pyysalo, 1975). The total amount of potassium iodate-reducing materials, calculated as ethylidene gyromitrin, in fresh central European *G. esculenta* is 1200–1600 mg/kg (List & Luft, 1968).

It has been proposed that ethylidene gyromitrin hydrolyses easily to methylhydrazine, which would be the toxic compound formed after *G. esculenta* has been eaten (Gray, 1972).

Cooked or dried *G. esculenta* has been reported to contain residues of hydrazones (List & Sundermann, 1974; Pyysalo, 1976; Schmidlin-Mészáros, 1974) and this has caused serious doubts concerning the mushroom's edibility.

Some preliminary animal tests on ethylidene gyromitrin have been reported (List & Luft, 1967; Mlodecki, Karkocha & Stec, 1962) but there are insufficient data to define the amounts that can be considered harmless. Preliminary tests with the higher homologues of ethylidene gyromitrin suggested that the toxicity of these compounds was somewhat lower than that of ethylidene gyromitrin (Pyysalo, 1975).

Franke, Freimuth & List (1967) have reviewed the food-poisoning outbreaks caused by *G. esculenta* in the period 1712–1965. According to these data, the most common clinical signs of intoxication in man are tiredness, nausea, vomiting, pain in the stomach and liver region, diarrhoea, increased body temperature, icterus, convulsions, dyspnoea, a rapid and weak pulse, the presence of protein, urobilinogen, urobilin and bilirubin in urine, coma and death. These signs are first seen 2–8 hr after ingestion of the toxin. At autopsy the most regular findings have been extensive fatty degeneration of the liver, kidneys and myocardium and haemosiderin deposits in the parenchymal cells of the liver, kidneys, spleen and bone marrow.

From data on food-poisoning outbreaks and on the content of potassium iodate-reducing materials in fresh false morel, it has been calculated that the possible lethal dose is 10–50 mg ethylidene gyromitrin/kg for children and 20–50 mg/kg for human adults (Schmidlin-Mészáros, 1975).

Previous studies of the toxicity of ethylidene gyromitrin in experimental animals (List & Luft, 1967; Mlodecki *et al.* 1962) have shown that an alcoholic extract of 600 mg *G. esculenta* was lethal for rabbits (List & Luft, 1967), that 180 g of the mushroom caused sickness in rats, and that 50 g false morel/kg body weight (90–180 mg potassium iodate-reducing material/kg) was lethal for dogs. S. Mäkinen (personal communication, 1976) showed that the LD_{50} of ethylidene gyromitrin was 70 mg/kg for rabbits and 320 mg/kg for rats.

The purpose of the present study was to examine the biological nature of the toxic effects of ethylidene gyromitrin, to assess the possible cumulative action, to investigate the variation between rabbits and chickens, to clarify the nature of the macro- and microscopic changes occurring and to determine the approximate dose level at which toxic effects occur.

EXPERIMENTAL

Test material. Ethylidene gyromitrin (acetaldehyde *N*-methyl-*N*-formylhydrazone) was synthesized according to the method described by List & Luft (1967), and the structure was confirmed by mass, infra-red and ^{1}H- and ^{13}C-nuclear magnetic resonance spectroscopy (Pyysalo, 1976). According to ^{1}H-nuclear magnetic resonance spectroscopy and gas–liquid chromatography (GLC) (50-m free-fatty-acid-phase glass capillary column, flame ionization detector) the purity of ethylidene gyromitrin was higher than 99%.

Animals were fed with ethylidene gyromitrin which had been stored at 4°C for 0–10 days after preparation. Gyromitrin solution in tap-water was prepared for the chickens every day and for the rabbits every third day. The stability of ethylidene gyromitrin in drinking-water was confirmed by GLC.

Animals and diet. New Zealand white rabbits provided by Jalokani, Kellokoski, Finland, were given pellets of Ewos Maintenance Feed of Rabbits and Guinea Pigs (Astra-Ewos, Sweden) and water *ad lib.* Animals were housed separately, in wooden cages with stainless-steel floors covered with peat, at 20 ± 2°C and 50–60% relative humidity. White Leghorn chickens, 2 days old, purchased from a local poultry farm (under continuous control of the area veterinary officer), were given Pikku-Tipu feed (Hankkija, Finland) to the age of 4 wk, and then Kasvatus-Tipu (Hankkija). Water was available *ad lib.* Male and female chickens were divided into four groups of 20 as shown in Table 2 and were housed to the age of 4 wk in a circular cage under infra-red lighting in a temperature which was decreased gradually from 40 to 20°C. Each group was then divided into four subgroups and housed five chickens to a cage under the same conditions as those for the rabbits.

Experimental procedure. Groups of five male and five female rabbits were given ethylidene gyromitrin in drinking-water at levels providing doses of 0 (control), 0·05, 0·5 or 5·0 mg/kg/day for 90 days. These dose levels were also given to the chickens. The consumption of feed and drinking-water was checked daily and the doses of drinking-water were adjusted so that a rabbit or a group of chickens consumed practically all the given dose. If necessary, untreated drinking-water was given in addition. The animals were weighed on day 0 and then every fortnight throughout the 90 days. The clinical condition of the animals was recorded daily. On the basis of the consumption of drinking-water and of the weight of the animals, the dose levels were controlled and adjusted weekly.

At the end of the 90-day period, surviving animals from all groups were killed by dislocation of the neck and then decapitated. Blood was taken from the jugular vein for haematological studies and serum analysis, and urine samples were collected from the urinary bladder. At autopsy all gross abnormalities were noted, and the heart, liver, spleen, kidneys and testes (chickens) were weighed. Samples of these organs and of the small intestine, adrenals, brain, lymph nodes, urinary bladder, gonads, uterus, skeletal muscle, bone marrow and any other tissues that appeared abnormal at autopsy were preserved in 10% buffered formalin. Paraffin-wax sections of these tissues were stained with haematoxylin and eosin for microscopic examination.

Table 1. *No. of deaths, main clinical observations before death and pathological findings in rabbits fed 0–5·0 mg ethylidene gyromitrin/kg/day for 90 days*

Findings	No. of rabbits affected in groups given doses (mg/kg/day) of			
	0	0·05	0·5	5·0
No. of male deaths	3	3	2	1
No. of female deaths	1	1	5	0
Clinical observations				
Weight loss	4	4	7	1
Anaemia	3	2	4	0
Inanition	4	4	7	1
Ear-mite infestation	1	2	2	0
Histopathological findings				
Intestinal coccidiosis	4	4	7	1
Myocardial degeneration	0	1	0	0
Myocarditis	0	0	2	0
Focal necrosis in liver	1	0	0	0
Parenchymal degeneration in liver	1	0	0	0
Chronic pericholangitis	0	1	5	1
Nutritional muscular dystrophy	0	0	2	0
Calcification of renal tubules	0	1	0	0
Non-purulent peribronchitis	1	0	0	0

Five male and five female rabbits were studied at each dose level.

Blood samples were examined for packed cell volume and for total and differential leucocyte counts. Serum taken from all the animals at autopsy was analysed for the activities of alanine aminotransferase (ALAT) and aspartate aminotransferase (ASAT) and for the content of urea nitrogen. The serum electrophoretic patterns were estimated by agarose-gel electrophoresis at pH 8·6 (Johansson, 1972). The electrophoretic patterns were traced densitometrically, and from the numerical data the percentage of each protein fraction was calculated.

Urine samples taken at autopsy were examined with Bili-Labstix (Ames Co., Slough, England) for semi-quantitative determination of protein, glucose, acetone, haemoglobin and bilirubin. The pH of all urine samples was measured, and the ethylidene gyromitrin content on day 90 following treatment at the highest dose level was determined using GLC.

RESULTS

All the chickens were healthy and behaved normally throughout the test period. Some of the rabbits, however, suffered from coccidial infections, typical signs of which were loss of appetite, lack of growth, weight loss, apathy and subsequent death. No treatment was given. All deaths occurred during the first month of the test (adaption phase), and no differences attributable to dosage were observed in the conditions affecting rabbits from the different groups (Table 1). Only one death occurred in the group receiving the highest dosage of ethylidene gyromitrin and no pathological changes attributable to treatment were found in the dead rabbits. The rabbits that survived the test period were, with one exception, clinically healthy and behaved normally. The exception was in the group receiving the highest dose of ethylidene gyromitrin and was shown to be suffering from chronic enteritis.

Weight changes occurring in the rabbits and chickens are shown in Table 2. Weight increase was significantly reduced ($P < 0.05$) in the 5-mg/kg group in both male and female rabbits, whereas there were no significant differences between different groups of chickens. Relative organ weights of the test animals are shown in Table 3. Significant differences ($P < 0.05$) were found in the relative weights of the livers of male and female rabbits in the 5-mg/kg group, and of male chickens in the 5-mg/kg group. No other significant differences in relative organ weights were observed.

No statistically significant differences were observed in the haematological examinations. In the clinico-chemical examinations, rabbits from the 5-mg/kg group exhibited a significant increase ($P < 0.05$) in serum ALAT and ASAT values, while chickens from the same dosage group and from the 0·5-mg/kg group showed an increase in ALAT. The urea-nitrogen levels in chickens from the 0·5- and 5-mg/kg groups were significantly ($P < 0.05$) increased to 0·65 and 0·50 mmol/litre, respectively, compared with 0·25 mmol/litre in the controls. No significant differences in the serum electrophoretic patterns were observed between different groups of either test animal.

From the urine samples of the rabbits, one case of mild proteinuria was observed in the control group, and two of moderate proteinuria in the 5·0-mg/kg group. Mild proteinuria was also observed in three animals from the 0·05-mg/kg group and in one from the 5·0-mg/kg group. Glucose was found in the urine of one rabbit from the 5·0-mg/kg group,

Table 2. *Body weight of rabbits and chickens fed 0–5·0 mg ethylidene gyromitrin/kg/day for 90 days*

Dose level (mg/kg/day)	Sex	No. of animals†	Mean body weight (g) at day 0	8‡	26	44‡	64	74	90	Weight gain (g) at day 90
Rabbits										
0 (control)	M	2	2280	2320	2300	2520	3038	3210	3405	1125
	F	4	2340	2530	2420	2562	2809	2957	3114	774
0·05	M	2	1850	1975	2070	2263	2408	2540	2677	827
	F	4	2112	2260	2137	2395	2645	2817	2959	847
0·5	M	3	2066	2170	2110	2273	2450	2443	2658	592
	F	—	—	—	—	—	—	—	—	—
5·0	M	4	2075	2135	2027	2255	2492	2512	2626	553*
	F	5	2270	2296	2263	2420	2600	2546	2590	320*
Chickens										
0 (control)	M	6	52	—	265	—	909	1283	1742	1690
	F	14	52	—	240	—	755	978	1275	1223
0·05	M	10	54	—	268	—	916	1306	1783	1729
	F	10	56	—	238	—	775	1026	1332	1276
0·5	M	10	54	—	261	—	893	1239	1693	1639
	F	10	53	—	245	—	745	1096	1341	1238
5·0	M	10	53	—	260	—	922	1279	1730	1677
	F	10	51	—	237	—	771	1007	1260	1209

†All animals surviving for 90 days were weighed. At each dose level five male and five female rabbits were started on the experiment. No chicken deaths occurred.

‡Body weights of chickens were not determined on days 8 or 44. Values of weight gain at day 90 marked with an asterisk differ significantly ($P < 0.05$) from those of the controls.

Table 3. *Relative organ weights of rabbits and chickens fed 0–5·0 mg ethylidene gyromitrin/kg/day for 90 days*

Organ	Sex	Relative organ weight (g/kg terminal body weight) in animals given doses (mg/kg/day) of			
		0 (control)	0·05	0·5	5·0
		Rabbits			
Heart	M	2·4	2·1	2·8	2·6
	F	2·4	2·2	—	2·7
Spleen	M	0·4	0·5	0·4	0·3
	F	0·4	0·4	—	0·4
Kidneys	M	5·1	6·1	5·9	5·8
	F	5·6	5·4	—	6·4
Liver	M	22·6	25·0	25·6	25·9*
	F	25·6	24·7	—	28·2*
		Chickens			
Heart	M	4·7	5·0	5·2	5·2
	F	4·3	4·4	4·5	4·0
Spleen	M	1·3	1·5	1·7	1·3
	F	1·8	1·6	1·8	1·6
Liver	M	17·4	18·1	17·8	19·3*
	F	19·2	19·4	20·1	19·0
Testes		1·1	3·8	2·2	2·1

Values are means for the numbers of animals shown in Table 2 and those marked with an asterisk differ significantly ($P < 0·05$) from those of the controls.

and haemoglobin in that of another. The urine of one rabbit from the 0·05-mg/kg group contained ketone bodies. Ethylidene gyromitrin was not detected in the urine of rabbits from the 5·0-mg/kg group, the detection limit of the method being 0·1 μg/ml.

Macroscopic and histological changes occurring in rabbits and chickens from the different groups are presented in Tables 4 and 5. The gross changes observed at autopsy were mostly minor and were randomly distributed between test and control groups.

Histological findings in the rabbits showed that the whole population had a subclinical coccidial infection, resulting in chronic pericholangitis. Degenerative changes were observed in the tubular cells in the kidneys of rabbits, particularly in the 0·5- and 5·0-mg/kg groups, but no other changes in the parenchymal organs could be related to the dose of ethylidene gyromitrin. The number of chickens with degenerative changes in the heart muscle was higher in all test groups than in the control group. The number showing degenerative changes in the liver parenchyma increased in the 0·5- and 5·0-mg/kg groups, and those with focal infiltrations of mononuclear cells in this tissue increased in the 5·0-mg/kg group. Degenerative changes in the tubular cells of kidneys were observed in greater numbers of individuals in the 0·5- and 5·0-mg/kg groups. Other histopathological changes (Table 5) were randomly distributed among the groups.

DISCUSSION

The acute toxicity of false morel to man has become apparent from numerous cases of food poisoning (Franke *et al.*, 1967), many of which have proved fatal. Chronic bronchial and skin reactions due to toxic compounds have also been observed in workers involved in the handling of these mushrooms (Bringhurst, Brine & Gershon-Cohen, 1959). It has been claimed that ethylidene gyromitrin taken orally may be a cumulative poison (Franke *et al.* 1967; Schmidlin-Mészáros, 1974), although information from animal tests on the effects of prolonged ingestion has not previously been reported. In the present work, total amounts of 4·5, 45 and 450 mg/kg were fed over a 90-day period. Definite biological effects were observed in rabbits only in conjunction with the highest dose level, which corresponded to 6·8 times the acute LD_{50} of 70 mg/kg, indicating that, at least in this species, ethylidene gyromitrin is only slightly cumulative. The results in chickens show that biological effects can be observed with a smaller daily dose (45 mg/kg in 90 days). As initial tests had shown that the LD_{50} for chickens was >400 mg/kg, this is a clear indication that chickens are more sensitive than rabbits to the accumulation of ethylidene gyromitrin.

Ethylidene gyromitrin was shown to have a significant ($P < 0·05$) effect on weight increase in rabbits of both sexes in the 5·0-mg/kg group. No significant differences in growth rate were detected between the different groups of chickens. As the average water and feed consumption rates of the test animals were independent of the dosage level, the variations in weight increase observed between the different groups of rabbits must be regarded as resulting from the dose administered.

Since an increase in the relative weight of the liver was limited to rabbits and male chickens receiving the highest dose, this effect must be interpreted as a result of the treatment. Enlargement of the liver has also been reported in cases of human poisoning (Franke *et al.* 1967). In addition, enlargement of the spleen was observed in two chickens in the two highest groups. This has also been found on post-mortem

Table 4. *Incidence of pathological findings in rabbits fed 0–5·0 mg ethylidene gyromitrin/kg/day for 90 days*

Pathological findings	No. of rabbits studied...	No. of rabbits affected in groups given doses (mg/kg/day) of			
		0	0·05	0·5	5·0
		6	6	3	9
Gross					
Ear-mite infestation (mild)		5	6	3	4
Intestinal coccidiosis		3	0	0	0
Dilatation of the right ventricle		0	1	0	0
Dilatation of the heart		0	0	0	1
Purulent pneumonia		0	0	1	0
Purulent pneumonia with abscesses (*Pasteurella multocida*)		0	1	0	0
Histological					
Degeneration of heart muscle		5	6	2	7
Chronic myocarditis		1	0	0	0
Myocardial infarction		0	1	0	0
Parenchymal degeneration in liver		4	3	2	4
Chronic pericholangitis		6	6	3	7
Degeneration in renal tubular cells		2	4	3	7
Calcification of renal tubules		4	2	0	4
Chronic interstitial nephritis		0	1	0	2
Non-purulent peribronchitis		2	2	0	3
Purulent bronchopneumonia		1	0	1	2
Skeletal muscle degeneration		1	0	0	1
Nodular hyperplasia of adrenal cortex		0	1	0	2
Chronic enteritis		0	0	0	1

Table 5. *Incidence of pathological findings in groups of 20 chickens fed 0–5·0 mg ethylidene gyromitrin/kg/day for 90 days*

Pathological findings	No. of chickens affected in groups given doses (mg/kg/day) of			
	0	0·05	0·5	5·0
Gross				
Enlargement of spleen	0	0	2	2
Pulmonary oedema	0	0	2	0
Petechical haemorrhage in sub-epicardium and sub-endocardium	1	0	1	1
Breast blister	0	0	1	0
Histological				
Myocardial degeneration	1	11	9	14
Intramural cardiac haemorrhage	0	0	2	1
Sub-epicardial haemorrhage	1	1	2	0
Focal infiltration of mononuclear cells in myocardium	2	4	4	4
Parenchymal degeneration in liver	4	6	9	10
Focal infiltration of mononuclear cells in liver parenchyma	11	12	11	17
Perilobular infiltration of mononuclear cells and eosinophils in portal tracts	5	8	6	8
Degeneration in renal tubular cells	2	4	8	8
Focal infiltration of lymphocytes in renal cortex or medulla	4	9	4	2
Focal infiltration of mononuclear cells in lung tissue	1	1	1	2
Pulmonary peribronchial and submucosal infiltration of mononuclear cells	1	2	1	1

examination of humans who have died after ethylidene gyromitrin poisoning (Franke *et al.* 1967). Significant differences in relative organ weights were not found in conjunction with acute poisoning in rabbits or rats (S. Mäkinen, personal communication, 1976).

In haematological examinations of samples taken randomly from the different groups, no differences attributable to the dose of ethylidene gyromitrin were observed, the variations being within the limits for normal animals. Thus the levels of gyromitrin administered did not affect the blood spectrum or the organs involved in the formation of blood.

In the clinico-chemical examinations, significant ($P < 0{\cdot}05$) increases in ASAT and ALAT values were observed in rabbits receiving the highest dose, indicating damage to tissues. The increased ASAT values suggest that this damage was extensive, as ASAT is located mainly in the mitochondria. Increase in ASAT values may result from damage to the liver, heart muscle or skeletal muscle. ALAT in cytoplasmic enzymes is released after comparatively low-level tissue damage. No differences which could be attributed to the dose of poison were detected in the serum enzymes of the chickens, but urea-nitrogen levels were significantly higher in chickens from the 0·5- and 5·0-mg/kg groups than from the control group. Although this increase was greater in the group on the lower dose it must be regarded as a result of the gyromitrin. The increased serum urea-nitrogen level indicates damage to kidney tissues, but the fact that the electrophoretic pattern of the serum proteins did not differ significantly between the dosage groups and the controls indicates that this damage was not extensive.

Semi-quantitative examination of the urine of rabbits from different groups showed a random appearance of clinico-pathological indicators. Bilirubin was not observed in the urine of any of the rabbits receiving ethylidene gyromitrin although it has been found at autopsy in the urine of rabbits given a single fatal dose (S. Mäkinen, personal communication) and together with other bile pigments and protein has been observed in humans suffering from acute poisoning (Franke *et al.* 1967). Haemoglobin was detected in the urine of one rabbit from the 5·0-mg/kg group and was not accompanied by inflammation of the lower urinary ducts. In cases of acute poisoning, haemoglobin was detected repeatedly in the urine of rabbits (Franke *et al.* 1967; S. Mäkinen, personal communication), and this was also true of human sufferers (Breuer & Stahler, 1966; Seeger & Weidmann, 1972). No other consistent variations were observed in the urine samples from the different groups.

Ethylidene gyromitrin could not be detected in the urine of animals from the 5·0-mg/kg group, although this might have been expected on the basis of preliminary experiments in which the unchanged toxin was detectable in the urine of rabbits at levels of 40 and 0·8 μg/ml on days 2 and 11, respectively, after administration of a single dose of 50 mg/kg. This preliminary test showed that ethylidene gyromitrin absorbed from the intestine did not degenerate completely to methylhydrazine, which is considered to be its toxic metabolite (Gray, 1972). The fact that ethylidene gyromitrin could not be detected in the urine of the test animals after treatment for 90 days may indicate that the rabbit is capable of adapting to and eliminating small repeated doses.

Degenerative changes in the tubular cells of the kidneys were observed to increase in proportion to the dose applied in both rabbits and chickens. Although it is difficult to define the extent of damage on the basis of histopathological examination, the increased levels of urea nitrogen found in chickens indicate that the degree of tissue damage was dose-dependent.

Szepietowski & Ratajczak (1971) have reported damage to kidney tissues following ingestion of false morel. According to Franke *et al.* (1967), degenerative changes in the renal tubular cells were not observed in human cases of ethylidene gyromitrin poisoning although at least some of the reported changes in urine indicated that degeneration in the kidneys had taken place.

Degeneration of the liver parenchyma did not occur more frequently in the experimental groups than in the control group but was more severe in the highest dosage group. Serum analyses gave no clear indication of damage to the liver; the increased ASAT and ALAT values indicated tissue damage, but could not be attributed directly to liver damage. Significant changes indicative of polyclonal gammapathia were not found in the serum electrophoretic pattern of the 5·0-mg/kg group, suggesting that the degenerative changes occurring in the liver were mild. In humans who had died from the toxic effects of false morel, fatty degeneration of liver has been a common finding (Franke *et al.* 1967). In chickens, histological evidence of degenerative changes in the liver parenchyma was found in conjunction with dose levels of 0·5 and 5·0 mg/kg/day. Focal infiltration of mononuclear cells into the parenchyma was also clearly greater in the 5·0-mg/kg group. However, the clinico-chemical findings for chickens do not give significant support to these histological observations.

Degenerative changes were observed in the heart muscle of chickens in all treated groups, but an increase in reparative changes was also evident. There were no significant differences in the relative weight of the heart in the different groups, and the clinico-chemical findings did not support the histopathological findings. On this basis it appears that the chickens had begun to compensate for the heart-muscle degeneration caused by intake of ethylidene gyromitrin. The mild degenerative changes in the heart muscle of the test rabbits cannot be regarded as a result of the ingestion of ethylidene gyromitrin and other changes in the hearts of the test rabbits must also be considered to be independent of treatment.

Pathological observations apart from those above (Tables 1, 4 and 5) are not clearly attributable to the effects of ethylidene gyromitrin. It is perhaps worth emphasizing that no evidence of increased susceptibility to tumour induction was observed as a result of the toxin. It has been suggested that ethylidene gyromitrin decomposes in the body to methylhydrazine, which has been shown (Thoth & Shimizu, 1973) to be carcinogenic. No experimental data concerning the metabolism of ethylidene gyromitrin in man or test animals has been published. No evidence of pathological changes in the central nervous system was found in the present experiments, but oedema has

been observed in the brains of human patients suffering from severe poisoning.

The rabbit deaths occurring during the experiment (Table 1) were shown to be the result of a coccidial infection, and showed no dependence on the level of toxin received. Although only one death occurred in the 5·0-mg/kg group, ethylidene gyromitrin can hardly be considered as a coccidiostat; the differences between the groups are almost certainly random.

This research indicates that the no-effect level of ethylidene gyromitrin is 0·5 mg/kg/day for rabbits and 0·05 mg/kg/day for chickens. Ethylidene gyromitrin is the main toxic component of false morel; higher homologues of this compound were shown in preliminary tests to have a lower level of toxicity (Pyysalo, 1975). It is suggested that the no-effect level of ethylidene gyromitrin for chickens should be further reduced by a safety factor of 1/100, giving an acceptable daily intake (ADI) for man of 0·0005 mg/kg/day. Thus for a 70-kg man, the maximum daily dose would be 0·035 mg or, assuming a daily consumption of 100 g of the prepared mushroom, an acceptable level of ethylidene gyromitrin of 0.35 mg/kg mushroom.

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THE EFFECT OF GARLIC EXTRACT ON THE ACTIVITY OF SOME ENZYMES

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Abstract—An extract obtained from garlic bulb (*Allium sativum*) was shown to inhibit hepatic and serum glutamic–oxalacetic transaminase, glutamic–pyruvic transaminase, lactic dehydrogenase and cholinesterase *in vitro*. The garlic extract stimulated the activity of liver adenosine triphosphatase in intact mitochondria, but had no effect on this enzyme after disruption of the mitochondria. The significance of these results is discussed.

INTRODUCTION

Garlic (*Allium sativum*) is widely used as a condiment and food. It has been used in folk medicine since ancient times and is still used in many parts of the world as a stimulant, antiseptic and diuretic and in pulmonary diseases and other infections (Watt & Breyer-Brandwijk, 1962). It has been suggested that it exerts some antibiotic activity against *Mycobacterium tuberculosis*, the growth of which was completely inhibited by a garlic juice concentration of 0·02%, even after boiling or prolonged storage of the juice (Rao, Rao & Venkataramon, 1946). The volatile substances emitted from fresh garlic paste are highly effective in the treatment of infected wounds and inhibit the growth of *Staphylococcus aureus* and *Brucella abortus* (Huddleston, Dufrain, Barrons & Giefel, 1944). Several investigators have demonstrated the presence of an antibacterial factor in garlic, and an active principle, known as allicin, has been isolated from the clove (Cavallito & Bailey, 1944; Cavallito, Buck & Sater, 1944; Johnson & Vaughn, 1969). This material is derived from alliin, (+)-*S*-allyl-L-cysteine sulphoxide, which is broken down by the enzyme, alliinase, present in the intact garlic clove. When the cells are damaged, alliinase breaks down alliin to allicin, pyruvic acid and ammonia (Stoll & Seebeck, 1951).

The mode of action of the active principle is not known, but of possible relevance are the effects of the active compound on some enzymes important in metabolism. Some of these effects are described in this communication.

EXPERIMENTAL

Preparation of mitochondria. Guinea-pigs were killed by decapitation and their livers were removed and placed on ice. Minced liver (10 g) was washed with cold 0·25 M-sucrose solution and liver mitochondria were prepared in the cold, according to the method of Johnson & Lardy (1967), the sedimented mitochondria being suspended in 10 ml 0·25 M-sucrose solution. The mitochondria were broken either by sonication on ice in an MSE 150 W Ultrasonic Disintegrator for 60 sec or by alternately freezing the mitochondrial suspension in a liquid-nitrogen bath and thawing it at room temperature, the freezing–thawing process being repeated three times.

Preparation of serum and hepatic enzymes. Blood taken from the cow, sheep or rabbit was allowed to clot in a water bath at 37°C for 30 min. The serum obtained was centrifuged at 1000 **g** for 5 min and the cell-free serum was kept on ice until used for the enzyme study. Whenever liver enzymes were used, a fresh guinea-pig liver was taken, cut into small pieces and washed with cold Tris-HCl buffer (0·005 M, pH 7·4). The tissue was then homogenized in 20 ml cold buffer in a Vortex homogenizer for 30 sec and subsequently in a glass teflon homogenizer. The homogenate was centrifuged at 100,000 **g** for 60 min and the resulting supernatant was used for the enzyme study.

Determination of adenosine triphosphatase (ATPase) activity. The activity of this enzyme was measured by the release of orthophosphate from ATP. The garlic extract (0–10 mg) was added to 1·0 ml of a reaction mixture consisting of 50 μmol Tris-HCl buffer (pH 7·5), 250 μmol sucrose, 10 μmol $MgCl_2$, 0·25 μmol EDTA and mitochondria, the volume of 1 ml being made up with water. The reaction was started by addition of 5 μmol ATP and was stopped, after incubation at 35°C in a metabolic shaker for 10 min, by addition of 2·0 ml 10% trichloroacetic acid. The mixture was then centrifuged at 5000 **g** for 10 min and inorganic phosphate was determined in aliquots of the supernatant according to the method of Fiske & Subbarow (1925).

Other enzyme assays. Glutamic–oxalacetic transaminase (GOT) and glutamic–pyruvic transaminase (GPT) were measured colorimetrically (Bergmeyer & Bernt, 1963). Lactic dehydrogenase (LDH) was measured spectrophotometrically by the disappearance of NADH at 340 nm (Bergmeyer, Bernt & Hess, 1963). Cholinesterase was measured spectrophotometrically by the breakdown of acetylthiocholine iodide and measurement at 405 nm of the colour complex formed with 5,5′-dithiobis-(2-nitrobenzoic acid) (Boehringer Mannheim GmbH, 1966).

Preparation of garlic extracts. Fresh garlic bulbs (100 g) were peeled and homogenized with 200 ml

Table 1. *Effect of fresh garlic extract on the activity of serum enzymes*

Extract added (mg/ml reaction mixture)	Enzyme activity (mU/ml)*			
	GOT	GPT	LDH	CE
0	86	36	540	286
0·5	57	28	—	160
1·0	44	23	480	149
1·5	34	21	454	109
2·5	26	12	406	89
5·0	16	7	329	37
10·0	13	4	227	—

GOT = Glutamic–oxalacetic transaminase
GPT = Glutamic–pyruvic transminase
LDH = Lactic dehydrogenase CE = Cholinesterase
*For identification of the methods used, see Experimental section.

cold double-distilled water in Waring blender, and the homogenate was centrifuged at 100,000 **g** for 60 min. The supernatant, 1 ml of which was equivalent to 50 mg dry matter, was used at once or kept frozen at −10°C until required. The effect of heat-treatment or dialysis on the activity was determined on this extract. For the former, the extract was heated in a boiling-water bath for 30 min and then centrifuged at 10,000 **g** for 15 min, the supernatant being retained for testing. Dialysis was carried out in the cold against 50 vols double-distilled water for 24 hr using a dialysis tube of pore size 2·4 nm.

Commercial diallyl sulphide and diallyl disulphide were obtained from Fluka AG, Switzerland, and dissolved for use in absolute ethanol to give a 20% solution which was added to the reaction mixture. The ethanol was then evaporated prior to addition of the enzyme.

RESULTS

Effect of garlic extract on serum enzymes

Extracts of garlic bulbs inhibited GOT activity, the degree of inhibition being dose-related and reaching nearly 90% with 10 mg extract/ml reaction mixture (Table 1). There was little or no difference in effect between the fresh and frozen extracts, and boiling the extract for 30 min did not affect its inhibitory capacity. The inhibitory factor was not dialysable. Similar results were obtained when GPT activity was determined in the presence of garlic extracts. These enzymes were unaffected, however, when either diallyl disulphide or diallyl sulphide was added to the reaction mixture.

Fresh, frozen or boiled garlic extract inhibited LDH activity, by up to 58% with 10 mg extract/ml reaction mixture. A similar level of inhibition (about 40%) was obtained with 5 mg of the extract (Table 1). Dialysed or heat-treated and dialysed extracts lost most of their original inhibitory capacity, and a capacity to inhibit the enzyme was found in the external water after dialysis whether or not the extract had been boiled. The effect of diallyl disulphide or diallyl sulphide on LDH could not be tested, since these sulphides oxidized non-enzymatically the NADH required for the assay of this enzyme.

Inhibition of cholinesterase reached 87% with 5 mg fresh or frozen garlic extracts in a concentration of 5 mg/ml reaction mixture (Table 1). Heating the extract at 100°C for 30 min resulted in a partial loss of this inhibitory capacity. Dialysis of the heated extract resulted in a further reduction in the inhibitory effect, inhibition by the heated dialysed extract being only 18% at 5 mg/ml.

Effects of garlic extract on liver enzymes

Similar results were observed with GOT, GPT, LDH and cholinesterase derived from liver. The garlic extract also contained a compound that affected the ATPase in liver mitochondria. Relatively low concentrations of fresh garlic extract in the reaction mixture caused an increase in ATPase activity (Table 2), addition of 1·0 mg fresh extract to 1 ml of reaction mixture resulting in an increase in activity of over 60%. Addition of higher concentrations resulted in lower levels of stimulation (Table 2). Addition of extract that had been heated at 100°C for 30 min resulted in a marked stimulation of ATPase activity, which reached a maximum (at almost three times the original level) on addition of 5·0 mg extract (Table 2).

Breaking the mitochondria by sonication or freezing and thawing increased the ATPase activity in extract-free preparations to a level almost as high as that of preparations of intact mitochondria treated with 5·0 mg boiled garlic extract. This initially high level of activity was not affected by addition of fresh or boiled extract to the mixture containing broken mitochondria (Table 2).

Table 2. *Effect of garlic extract on the activity of adenosine triphosphatase from intact and broken mitochondria*

Extract added (mg/ml reaction mixture)	P_i liberated (nmol/mg protein/10 min)		
	Intact mitochondria		Broken mitochondria
	Fresh extract	Boiled extract	Fresh extract
0	800	800	2360
0·5	1100	1360	2390
1·0	1300	1480	2340
1·5	1240	1610	2310
2·5	1100	1840	2240
5·0	1020	2220	2200
10·0	920	2010	2160

For details of the method, see Experimental section.

DISCUSSION

The garlic-extract components that affected the activity of the serum enzymes have not been identified. It seems, however, that several different substances in the garlic bulb are involved and that these differ in their physical characteristics.

The factor that inhibited the transaminases (GOT and GPT) was heat-stable, remaining in the extract after heating at 100°C for 30 min. Furthermore, it had a relatively large molecular weight, since it did not dialyse through a pore size of 2·4 nm. Diallyl disulphide, which is found in garlic extract (Stoll & Seebeck, 1951), was not responsible for the inhibition, since addition of this substance to the reaction mixture had no effect on the enzyme activity. The substance that inhibited LDH was different from the one that inhibited the activity of GOT and GPT, since it was heat-stable but also dialysable. The cholinesterase-inhibiting factor was heat-labile, the inhibitory capacity of 5 mg of heated extract added to the reaction mixture being almost 50% less than that of the unheated extract. After both heating and dialysis, cholinesterase-inhibiting ability was lost almost completely.

The ATPase-stimulating compound in garlic extract appears to act only in preparations of intact mitochondria since, while a significant stimulation was shown with intact mitochondria, no significant effect was evident in the presence of broken mitochondria. It is possible that this compound facilitates the transport of ATP inside the mitochondria where the ATPase enzyme is located. Once the mitochondrial membrane had been broken by sonication or freezing and thawing, ATP could easily reach the site of enzyme action and therefore no stimulation was observed when garlic extract was added (Table 2). Breaking of the mitochondrial membrane increased the activity of this enzyme, the resulting level of activity being higher than that in intact mitochondria in the presence of untreated garlic extract. The higher levels induced in intact mitochondrial preparations by addition of boiled garlic extract were similar to those obtained following sonication of the mitochondria.

These results show that the crude extract obtained from garlic contains several factors that, on the one hand, inhibit some enzymes and, on the other, affect biological membranes. The toxicity of these factors to people who eat garlic requires further investigation. Garlic extract is known to contain antibacterial factors but further study is needed to determine whether this antibacterial action is the result of enzyme inhibition or membrane disruption.

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REPRODUCTIVE AND PERI- AND POSTNATAL STUDIES WITH HEXACHLOROPHENE

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Abstract—Hexachlorophene (HCP) was evaluated for its effects on reproductive performance and perinatal and postnatal development in rats. To evaluate the effects on fertility and general reproductive performance, 5 or 10 mg HCP/kg/day was given to males for 63 and females for 14 days prior to mating. Dosing was continued until day 14 of gestation for half of the females and until after weaning for the other half. Mating and fertility were not affected, and progeny were delivered in normal numbers, were free of structural abnormalities and developed normally throughout lactation. A slight reduction in pup survival was observed at the 10 mg/kg dose level. Treatment of females with either 15 or 30 mg HCP/kg/day during the last third of gestation and until weaning caused reductions in the body weights and survival of the progeny. Reduced body-weight gain and some deaths were encountered among the maternal animals treated with 30 mg HCP/kg. At this level, an increase in stillbirths was noted, although all progeny, both stillborn and viable, were structurally normal.

INTRODUCTION

Hexachlorophene (2,2′-methylenebis-(3,4,6-trichlorophenol); HCP) has been used as an antibacterial agent in a wide variety of products, including topical disinfectants, pre-operative preparations and surgical scrubs (Clark, Lockwood & Lewit, 1947; Havens, Benham & Clark, 1957; Reber, Bircher & Grumback, 1960).

The finding of toxic effects, including convulsive seizures in man (Herter, 1959; Larson, 1968) and functional effects, hindquarter paralysis and histopathological changes in the brain and spinal cord of rats (Kennedy, Dressler, Richter, Keplinger & Calandra, 1976; Kimbrough & Gaines, 1971) prompted evaluation of the possible effects of the chemical on reproductive processes.

Thorpe (1967) found that oral administration of HCP to male rats and sheep produced degeneration of spermatogenic cells, and Gaines & Kimbrough (1971) found reduced survival in the F_1-generation offspring of rats fed 100 ppm. In contrast, no signs of impaired reproduction were seen over three generations of rats fed HCP at levels up to 50 ppm (Kennedy, Smith, Keplinger & Calandra, 1975a), and Alleva (1973) found that HCP did not interfere with hamster reproduction.

The teratogenic response of rabbits and rats to HCP given orally at dose levels closely approaching those resulting in maternal death is considered minimal (Kennedy, Smith, Keplinger & Calandra, 1975b), but four rats fed 500 ppm during gestation produced offspring with cleft palate (Oakley & Shepard, 1972), and the introduction of HCP into the vagina of pregnant rats produced a variety of foetal malformations (Kimmel, Moore, Hysell & Stara, 1974).

The study reported here was undertaken to evaluate the effects of daily oral doses of HCP on both the reproduction and the peri- and postnatal performance of rats.

EXPERIMENTAL

Materials. HCP (Hexachlorophene, G-11 brand) was supplied by Givaudan Corp., Clifton, N.J.

Animals and diet. COBS random-bred albino rats were received at these laboratories either at 21 days of age or after impregnation at the Charles River Breeding Laboratories, Wilmington, Mass. The day of sperm-positive vaginal examination was designated gestation day 0. All animals were housed individually in hanging stainless-steel cages and were maintained on a standard powdered feed, obtained from Ralston-Purina, St. Louis, Mo., and water *ad lib.*

Experimental design. Preliminary range-finding studies were conducted in both male and female rats to determine tolerable doses. In the reproduction study, animals were treated at dose levels of 0 (control), 5 or 10 mg HCP/kg/day. The material was administered by gavage as a 3% suspension in 1% aqueous methylcellulose, the control animals receiving the vehicle alone in a volume corresponding to that given to the high-dose animals. Males received the test material 63 days before mating and until mating was accomplished; females received the test material 14 days before mating and until the weaning of the litter at 21 days of age. Each group consisted of 10 males and 20 females. At 100 days of age, females were caged in pairs and mated with a male, the exact day of copulation being determined by the presence of spermatozoa in the vagina. Males of a given treatment group were rotated with females at 10-day intervals (two female oestrous cycles) and each female received a maximum of three different males. Daily observations were made on all animals for death and for any significant behavioural effects, and each animal was weighed weekly. Effects on fertility, mating behaviour, pregnancy, length of gestation, and lactation performance were monitored. At day 14 of gestation, half the female animals from each group were killed, and a gross examination of the ovaries,

Table 1. *Effects of HCP on reproductive parameters in rats*

Dose (mg/kg)	Mating index*	Male fertility index†	Female fertility index‡	Fecundity index§
0	57·1	80·0	100·0	100·0
5	82·6	100·0	94·7	90·0
10	42·2	100·0	94·7	90·0

*(No. of copulations/no. of oestrous cycles required) × 100.
†(No. of sires/no. of males mated) × 100.
‡(No. of pregnancies/no. of females mated) × 100.
§(No. of pregnancies/no. of copulations) × 100.

uteri and foetuses were conducted. The remainder of the females continued on HCP treatment until the offspring were delivered and weaned. Progeny were weighed at delivery, and a complete examination for external defects was conducted. The body weights of all pups were recorded on days, 1, 4 and 21 after birth.

In the peri- and postnatal study, groups of 20 pregnant rats were treated orally with 0 (control), 15 or 30 mg HCP/kg/day from day 15 of gestation and throughout lactation. The parameters examined were maternal body weight (periodically throughout), deaths and behaviour, length of gestation, and progeny number, deaths, behaviour, survival indices and body weight (on days 0, 1, 4 and 21 after birth).

Statistical analyses were conducted on all data using either a one-way analysis of variance and *t* test or chi-square analysis.

RESULTS AND DISCUSSION

In the preliminary study, rats exposed to either 20 or 30 mg HCP/kg for 3 consecutive weeks displayed significantly reduced weight gains. In the reproduction study, doses of 5 and 10 mg/kg were used and in the peri- and postnatal study, in which treatment for only 4 wk was required, higher doses (15 and 30 mg/kg) were selected.

No significant changes were observed in either body weight or overall weight gain of males or females treated with 5 or 10 mg HCP/kg during the reproduction study. No deaths occurred, and all animals were free of outward signs of a pharmatoxic response to the chemical. Reproductive parameters (Table 1) indicated no adverse response to treatment

Table 3. *Survival indices of progeny from rats exposed to HCP*

Dose (mg/kg)	Live birth index†	Survival index‡ at day 1	4	12	21
Reproduction study					
0	96·5	99·1	99·1	95·5	92·7
5	95·9	97·4	91·4	87·1	87·7
10	98·0	95·9	95·9	86·6	83·5*
Perinatal and postnatal study					
0	100·0	100·0	100·0	100·0	93·1
15	96·9	95·1	79·2**	77·8**	64·6**
30	90·2*	72·3**	58·0**	55·5**	38·7**

†(No. of viable pups delivered/total no. of pups delivered) × 100.
‡(No. of viable pups at stated time/no. of viable pups delivered) × 100.
Values marked with asterisks differ significantly. (*$P < 0.05$; **$P < 0.01$) from the corresponding control value.

with HCP. At the time of the interim examination (day 14 of gestation), test and control animals exhibited similar numbers of corpora lutea, implantation sites, resorption sites and viable foetuses (Table 2). The apparent increase in resorption sites among animals in the 10 mg HCP/kg group resulted from two females showing, individually, five and four resorption sites.

The number of both viable and stillborn pups delivered to HCP-treated mothers was similar to that of the controls (Table 2), and all pups delivered were free of gross structural abnormalities. The number of pups surviving the later stages of lactation was reduced among animals treated with 10 mg HCP/kg and was reflected in the lowered 12- and 21-day survival indices (Table 3). This was not observed among the animals exposed to 5 mg HCP/kg.

Although the body weights of pups from both test groups early in lactation were lower than those of the concurrent controls (Table 4), the values obtained were well within the normal range for rats of this strain. Furthermore, total body-weight gains during the lactation period indicated no significant differences between pups derived from control and from HCP-treated females.

Treatment of female rats with 30 mg HCP/kg from day 15 of gestation resulted in maternal toxicity. These animals lost an average of 7 g from day 15 to day 21 (compared to the usual weight gain of 10–15 g). Two animals displayed weakness of the

Table 2. *Effect of HCP on reproductive performance in rats*

Dose (mg/kg)	Interim examination: Corpora lutea	Implantation sites	Resorption sites	Viable foetuses	Natural delivery: Viable pups delivered	Stillborn pups	Viable pups on day 4	Viable pups on day 12	Viable pups on day 21
0	13·1	12·8	0·4	12·4	11·0	0·4	10·9	10·5	10·1
5	14·2	13·8	0·8	13·1	12·8	0·6	11·7	11·2	10·3
10	14·3	13·2	1·4*	11·7	10·8	0·2	10·1	9·3*	8·4*

Values are means for 20 females and those marked with an asterisk differ significantly ($P < 0.05$) from the corresponding control value.

Table 4. *Body weights of progeny from rats exposed to HCP*

Dose (mg/kg)	Mean body weight (g) on day				
				21	
	0	1	4	Male	Female
Reproduction study					
0	6·3	7·0	10·6	42	39
5	6·0*	6·5*	9·3*	37	38
10	6·0*	6·4*	9·0*	41	40
Perinatal and postnatal study					
0	6·8	7·1	11·4	42	41
15	5·8**	6·1**	8·9**	41	38
30	5·1**	5·5**	8·1**	36**	38

Values marked with asterisks differ significantly (*$P < 0{\cdot}05$; **$P < 0{\cdot}01$) from the corresponding control value.

hindlimbs and severe hypoactivity, became comatose following two or three doses and were sacrificed *in extremis*. The remaining animals in this group showed no signs of a pharmacotoxic response, but body weights throughout lactation remained 4–6% lower than those of the corresponding controls. Animals treated with 15 mg HCP/kg did not display any adverse reactions during the dosing period.

Among the animals given 30 mg HCP/kg, the number of viable pups delivered decreased and the number of stillborn pups increased (Table 5). The number of pups/female throughout lactation was reduced at this level, and animals given 15 mg HCP/kg showed reduced pup numbers from lactation day 4 to weaning. Survival indices (Table 3) reflect these lowered number of pups/female. All progeny were externally normal, and the cause of death in these animals was not apparent; no pharmacological signs were found in any of the progeny. At birth and on lactation days 1 and 4, a dose-related decrease in pup weight was observed (Table 4). At weaning, the only significant decreases in body weight were among male offspring of the animals given 30 mg HCP/kg. Body weights of weanlings from small litters tend to be greater than those from large litters, but although there were fewer viable weanlings/litter in the treated groups, the weaning body weights of both sexes in the 15 mg HCP/kg group and of female progeny in the 30 mg HCP/kg group were essentially the same as or slightly lower than those of the controls.

No signs of interference with reproductive processes were detected in rats given either 5 or 10 mg HCP/kg before and during the weaning of a single litter, although a slight reduction in progeny survival was suggested among the 10 mg HCP/kg group. All progeny delivered were structurally normal and showed no outward signs of a pharmacological response to HCP. When either 15 or 30 mg HCP/kg was given during the last third of gestation and

Table 5. *Effects of HCP on perinatal and postnatal performance in rats*

Dose (mg/kg)	Number of viable pups delivered	Number of stillborn pups	No. viable pups on day			
			1	4	12	21
0	9·2	0·0	9·2	9·2	9·2	8·5
15	9·6	0·3	9·1	7·6*	7·6*	6·2**
30	8·5	1·0**	6·1**	4·9**	4·7**	3·3**

Values are mean numbers of pups/female and those marked with asterisks differ significantly (*$P < 0{\cdot}05$; **$P < 0{\cdot}01$) from the corresponding control value.

through the lactation period, a reduction in the size of the progeny and in their survival was observed. At the highest level, maternal toxicity was indicated by reduced body weights and some deaths.

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THE EFFECT OF SURFACTANTS UPON RAT PERITONEAL MAST CELLS *IN VITRO*

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Abstract—Rat peritoneal mast cells incubated with pure surfactants released the histamine stored in the cell granules. Histamine release was not accompanied by degranulation, but was associated with degeneration of the cell membrane and cytoplasm. The concentrations of individual surfactants required to release histamine depended not only upon the nature of the hydrophilic moiety, the alkyl sulphates being particularly effective, but also upon the length of the alkyl chain, with the lauryl and myristyl moieties being the most potent. The non-ionic ethoxylated long-chain alcohols were generally as effective as the corresponding alkyl sulphate.

INTRODUCTION

The tissue mast cells, by virtue of their large stores of vaso-active amines such as histamine and 5-hydroxytryptamine (Keller, 1969; Uvnäs, 1969), are important in the mediation of cutaneous inflammation evoked by a wide range of stimuli, such as infection, physical and chemical trauma and antigens. The role of histamine, in particular, as a mediator of inflammation has been widely studied, as reviews by Wilhelm (1973) and Willoughby (1973) indicate, and the mechanism of its release from the mast cell has been elucidated for certain specific stimuli, such as compound 48/80, a condensation product of *p*-methoxyphenethylmethylamine with formalin (Lagunoff, 1972; Röhlich, Anderson & Uvnäs, 1971), allergens (Bach, 1974), hypo-osmolarity, certain cytotoxic chemicals, drugs and venom (Bloom & Haegermark, 1967; Frisk-Holmberg, 1971; Jansson & Penttilä, 1969). Most of these studies have utilized isolated mast cells, harvested from rat peritoneal washings, *in vitro*.

Surface-active materials (surfactants) belong to a class of compounds that may cause primary cutaneous inflammation (Lansdown, 1972), especially when applied in high concentration to the skin of laboratory rats (Prottey & Ferguson, 1975), and the way in which they release histamine from mast cells is thought to be similar to that of some chemicals and lipophilic drugs (Röhlich *et al.* 1971). As surfactants embody a whole range of chemical compositions, ranging from simple carboxylic acid soaps to complex synthetic detergents, this study was performed to investigate the relationship between surfactant structure and the potential for releasing histamine from peritoneal mast cells isolated from the rat.

EXPERIMENTAL

Animals and materials. All rats were of the Colworth Wistar albino strain, weighed about 200 g and were fed Spital diet and water *ad lib.* Alkyl sulphates were obtained from Kodak Ltd., London. Caprylic (C_8), capric (C_{10}), lauric (C_{12}), myristic (C_{14}), palmitic (C_{16}) and stearic (C_{18}) acids (all more than 98% pure) were purchased from Koch-Light Ltd., Colnbrook, Bucks., and conversion to sodium soaps was effected by dissolving the acid in mixtures of alcohol and water and neutralizing to pH 8–9 with stoichiometric quantities of sodium hydroxide. The resultant waxy products were freeze-dried after removal of alcohol by flash evaporation. All other surfactants were synthesized in our laboratory by Mr. C. T. James:

> For alkyl isethionates, the appropriate pure carboxylic acid was converted to the acid chloride with thionyl chloride, and then purified by distillation. The acid chloride was then reacted with sodium isethionate and the resulting isethionate ester was purified by recrystallization. Long-chain (C_8–C_{18}) primary alcohols, all more than 95% pure, were purchased from Koch-Light Ltd., and the tosyl derivatives were prepared using *p*-toluenesulphonyl chloride. Mono-, tri- and hexaethoxylate derivatives were formed by condensing individual tosylates with monosodium ethylene glycol, monosodium triethylene glycol and monosodium hexaethylene glycol (the purity of the mono-, tri- and hexaethylene glycols first being ensured by distillation). The resulting alcohol ethoxylates were purified by distillation, solvent extraction, crystallization or chromatography. Absence of unreacted alcohols was always confirmed. Sulphated derivatives of the alcohol ethoxylates were prepared with chlorosulphonic acid, and the absence of unsulphated material was checked by chromatography. The surfactants studied were the C_8–C_{18} alkyl carboxylates, isethionates, sulphates, mono-, tri- and hexaethoxylates and mono- and triethoxy sulphates.

Full details of these methods will be published elsewhere (C. Prottey, T. F. M. Ferguson and C. T. James, in preparation 1976).

Preparation of rat peritoneal mast cells. The method of Perelmutter & Khera (1970) was followed. Rats were killed by CO_2 asphyxiation and their peritoneal

cavities were irrigated with 25–30 ml medium 199 (Morgan, Morton & Parker, 1950) containing 0·5 mg ethylenediaminetetraacetic acid (EDTA)/ml. The cells were harvested by centrifugation at room temperature at 250 g for 10 min and then re-suspended in a suitable volume of a buffered salt solution (154 mM-NaCl, 2·7 mM-KCl and 0·9 mM-$CaCl_2$) containing 10% (v/v) Sörensen's buffer (67 mM-Na_2HPO_4 + KH_2PO_4, pH 6·8). Mast cells were identified by staining with toluidine blue and counted in a Neubauer Chamber. It was estimated that mast cells comprised about 5–10% of the total peritoneal-cell population. In early studies, 10% rat serum was added, but this was later discontinued.

Extraction of histamine liberated during mast-cell incubations. Mast cells were incubated in a total volume of 1·0 ml of the buffered salt solution containing surfactant for 3 hr at 37°C, after which they were centrifuged and the supernatant solutions were diluted with an equal volume of 0·8 M-$HClO_4$; portions were then taken for histamine determination. The cell pellets were resuspended in 1·0 ml distilled water, were frozen and thawed three times and were then diluted with 1·0 ml 0·8 M-$HClO_4$ prior to histamine determination. Histamine was determined spectrofluorimetrically by the method of von Redlich & Glick (1965) using a Locarte spectrofluorimeter.

Critical micelle concentration (CMC). This was determined for the surfactants in the incubation buffer at 22°C using a du Nuoy Tensiometer.

RESULTS

Effect of serum on histamine release by surfactants

In all preliminary experiments with mast cells *in vitro*, 10% rat serum was added to the incubation medium in order to simulate more closely a physiological milieu for the cells. It was found that in the absence of added serum the level of spontaneous histamine release was usually higher, in some cases over 25% of the total cellular histamine being released in the absence of any surfactant during a 3-hr incubation. However, when the lytic action of surfactants was studied, it was anticipated that the presence of exogenous serum protein would cause a preferential binding of the surfactants, thereby lowering their effective concentration, and so the action of serum in a typical experiment on surfactant-induced lysis was examined. Figure 1 summarizes the data obtained.

There was no difference in the release of histamine by 0·1 mM-sodium caprate whether serum was present or not, but there was evidence that serum inhibited histamine release by 1·0 mM-caprate, 10% serum being more effective in this respect than 1%. The maximum amount of mast-cell histamine released by 1·0 mM-sodium caprate in the absence of serum was 30% and the level of spontaneous release in the absence of surfactant was 18%, which was reduced by addition of serum, indicating that serum protein protected mast cells *in vitro*. The effects of sodium laurate were similar to those of sodium myristate but were unlike those of sodium caprate. In the absence of serum, 1·0 mM-sodium laurate released 77·5% of the available histamine and 1·0 mM-sodium myristate released 80·5%. At 1% serum concentration these values were reduced

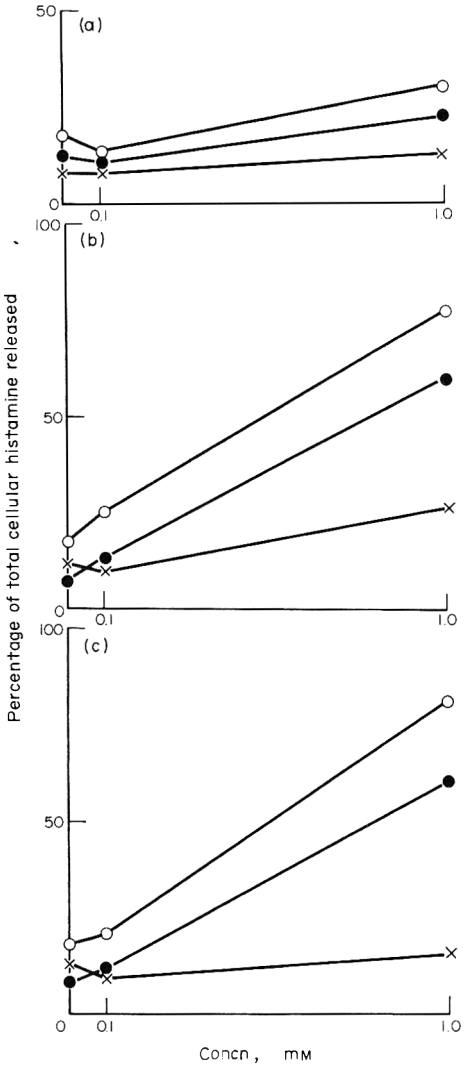

Fig. 1. Histamine release from rat peritoneal mast cells incubated *in vitro* with (a) sodium caprate, (b) sodium laurate and (c) sodium myristate in the presence (1%, ●; 10%, ×) and absence (○) of rat serum. Each 1·0-ml incubation contained 158,000 mast cells in buffered salt solution and was maintained for 3 hr at 37°C.

to 60%, whereas at 10% the degree of histamine release was 25% for laurate and 15% for myristate, values not dissimilar to the levels of spontaneous release. With 0·1 mM concentrations of these surfactants only incubations devoid of serum showed a release of histamine above the levels of spontaneous release. Not only did this experiment show that these two surfactants were more potent than sodium caprate in releasing histamine, but it was also clear that serum interfered with this ability, presumably by neutralizing their cytolytic effects by strong protein–surfactant interactions. Therefore, in all subsequent experiments serum was omitted from incubation media so that differences between individual surfactants could be studied.

Reproducibility of the surfactant lysis method

It was not possible, from one experiment to another, to harvest constant numbers of mast cells

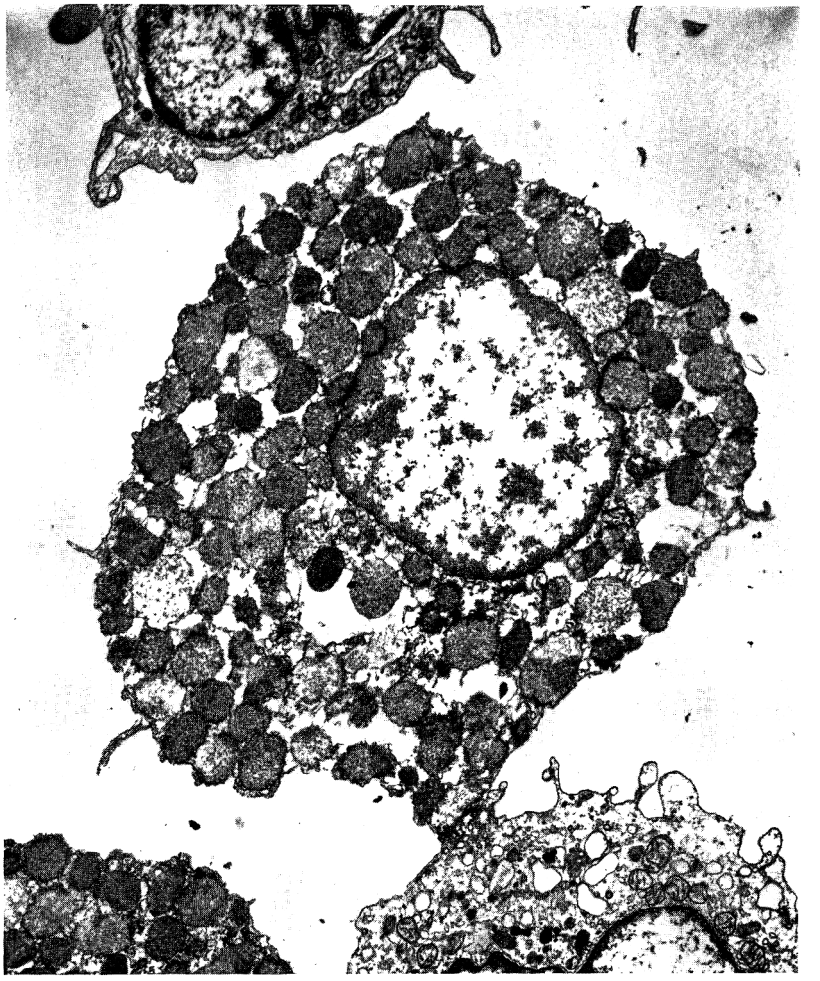

Fig. 2. Electron micrograph of rat peritoneal mast cells, incubated in the presence of 2·5 mM sodium lauryl sulphate and 10% rat serum. × 6500.

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Table 1. *Reproducibility of histamine release by sodium laurate*

Rat no.	Mast-cell concn (no. of cells/ml)	Histamine release (% of total mast-cell content) by surfactant added in concn (mM) of				
		0	0·1	0·5	0·75	1·0
1	175,000	29·4	14·4	69·9	74·1	85·6
2	100,000	15·2	12·6	69·1	61·5	60·9
3	87,500	11·6	18·4	65·1	65·4	70·9
4	200,000	7·7	10·2	61·1	65·0	71·0

Cells were incubated for 3 hr at 37°C in a total volume of 1·0 ml buffered salt solution containing no serum.

by peritoneal lavage. Also, although the exact number of mast cells incubated in a typical experiment was not constant from one experiment to another, the number used was generally of the order of 1×10^5/incubation. As a check on reproducibility, peritoneal cells were prepared from each of four rats and the individual preparations were found to contain 175,000, 100,000, 87,500 and 200,000 cells/ml. Aliquots from each were incubated with various concentrations of sodium laurate (with no added serum) and the resultant release of histamine is shown in Table 1. In all four cases there was no release of histamine greater than the level of spontaneous release with levels of sodium laurate below 0·5 mM, at which concentration between 60 and 70% of the total histamine was liberated. The experiment showed that at a concentration of sodium laurate between 0·1 and 0·5 mM the mast cells released the bulk of their stored histamine, and that increasing the surfactant concentration above 0·5 mM did not release appreciably more. Preparations of cells from four individual rats and containing widely differing numbers of mast cells gave similar results.

The nature and mechanism of mast-cell degranulation by surfactants

Mast cells were examined in the light microscope under various conditions. In distilled water almost all were lysed, many appearing with a 'halo' of granules around the exterior cell membrane. In buffer alone, the majority of the mast cells were rounded and packed with basophilic granules, although a small percentage showed signs of spontaneous degranulation. The situation with surfactants was different, however. Mast cells exposed to sodium laurate at a level below 0·1 mM were normal, but at higher concentrations there was evidence of degranulation or, more precisely, cell lysis in which the cells were denatured. Some of the liberated granules dissolved, while others appeared to coagulate at the plasma membranes. Such cells showed obvious signs of damage.

In a separate experiment, mast cells were exposed to 2·5 mM-sodium lauryl sulphate in the presence of 10% serum and were examined by electron microscopy. These conditions had previously been found to cause release of all available histamine. Although mast cells comprised less than 10% of the total cells present in the washings, they were the only ones to show signs of damage. A typical example is shown in Fig. 2. Most of the mast-cell granules were pale, diffuse and expanded, unlike the dense, darkly-staining granules seen in normal, intact rat mast cells *in vitro*. Their plasma membranes were absent or ruptured and the normally numerous microvillous projections were mostly absent. Cytoplasmic organelles were damaged or absent, and in particular the mitochondria were shrunken and there was vacuolation. Nuclei showed loss of chromatin. It thus appeared that surfactants had a denaturing effect upon mast cells, physically disrupting the cell membranes from which the granules would spill out and then release their histamine by ion-exchange with extracellular cations. The macrophages, eosinophils, lymphocytes and other cells also present in the peritoneal washings appeared normal.

Concentration dependency of mast-cell degranulation by surfactants

Mast cells were incubated with increasing concentrations of two pure surfactants, sodium lauryl sulphate and sodium lauroyl isethionate, and the release of histamine was measured and plotted in Figs 3a and 3b respectively. For both, it was found that histamine was released over very narrow and precise concentration ranges, which were consistent in replicate determinations. It was necessary for serum to be omitted from all of these incubations, however, as its presence greatly altered the concentration at which release of histamine occurred (Fig. 1). At all concentrations below the specific level, histamine release was always of the same order as the spontaneous release seen in control incubations without surfactants, being generally up to about 20%. It was never possible, however, to release all of the available mast-cell histamine with surfactants. The upper limit was generally between 60 and 90%, dependent upon each particular cell preparation. Presumably that proportion of mast-cell histamine that was never solubilized after surfactant treatment was bound to granules that were still within the cell and were possibly immature and unable to release their histamine by an ion-exchange.

Histamine release by surfactants of various structures

A wide range of pure surfactants, which had been either purchased or synthesized in our laboratory, was examined. In particular, the effect of length of the alkyl chains and the nature of the polar head groups were studied. In each case, the concentrations that caused specific release of histamine exactly as shown in Fig. 3(a,b) were determined and these are recorded in Table 2. With the alkyl carboxylates, alkyl isethionates and alkyl sulphates, the C_{12} (lauryl) moiety was the most potent at releasing histamine, as higher and lower homologues required a higher concentration. The C_{14} (myristyl) moiety was more

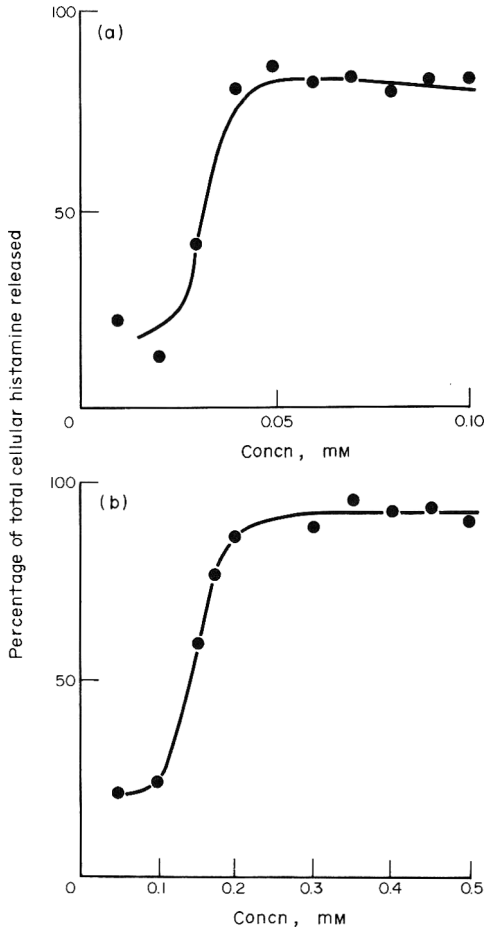

Fig. 3. Concentration curves for surfactant-induced release of histamine from rat peritoneal mast cells *in vitro*. Preparations of 89,000 mast cells in 0·9 ml buffered salt solution containing no rat serum were incubated for 3 hr at 37°C after addition of (a) sodium lauryl sulphate or (b) sodium lauroyl isethionate in 0·1 ml water.

effective for the ethoxylated surfactants. There were distinct differences between the various head groups of the anionic surfactants: alkyl sulphates were more potent than isethionates and carboxylates in that order, but the ethoxylate sulphates were uniformly similar to their parent alkyl sulphates. The non-ionic ethoxylates, especially the tri- and hexaethoxylates were unexpectedly effective at releasing histamine. These surfactants are neutral (i.e. unchanged) but possess bulky hydrophilic head groups, and they were generally as effective as the corresponding alkyl sulphates in releasing histamine.

Table 2 also shows the CMC of most of the surfactants in buffer. Although all mast-cell incubations were performed at 37°C it was not possible to measure CMC values at other than ambient temperature (22°C). These values would, if anything, be higher at 37°C. Nonetheless, it was observed that the concentrations of surfactant required to cause specific release of histamine were all below the CMC, at which surfactants would exist in solution as single molecules. Thus, the action of surfactants upon mast cells *in vitro* to cause histamine release must be a direct interaction and not the result of solubilization of membrane components in a surfactant micelle.

DISCUSSION

Total mixed rat-peritoneal cells were used throughout this study, and although the exact proportion of mast cells among these was never routinely determined, this was estimated to be less than 10%. It was assumed that the other major cell types of the peritoneal washings (macrophages, eosinophils and lymphocytes) contained little histamine, and that therefore the amounts of this amine released by various surfactants were derived from the mast cells.

In all experiments with surfactants, the release of histamine was observed to occur over a relatively narrow concentration range (Fig. 3), below which there was always only a low background release generally of about 10–15% of the total cellular histamine. This amount of histamine was always spontaneously released, even in the absence of any surfactant, and was due, presumably, to mechanical disruption of some of the mast cells during pipetting and other treatment. Performing surfactant incubation at 4°C did not reduce the amount of histamine lost in this way. This observation has been reported by others.

Table 2. *Concentrations of pure surfactants required to release histamine from rat peritoneal mast cells* in vitro

Polar head group	Alkyl chain length....	Histamine-releasing concn* (mM)					
		C_8	C_{10}	C_{12}	C_{14}	C_{16}	C_{18}
Carboxylate		>5·0† (15)	1·0 (1·3)	0·4 (10·0)	0·5 (6)	1·0 (IS‡)	ND
Isethionate		ND	0·76 (6·5)	0·15 (1·2)	c.0·5 (ND)	ND	ND
Sulphate		ND	0·5 (9·0)	0·03 (1·0)	0·025 (2·5)	0·5 (IS‡)	0·5 (IS‡)
Monoethoxylate		>1·0† (WS)	0·2–0·5 (1·0)	0·2–0·5 (0·1)	>1·0† (0·013)	IS	IS
Monoethoxy sulphate		>1·0† (WS)	0·5 (1·0)	0·05 (0·15)	ND	ND	ND
Triethoxylate		0·5 (WS)	0·075 (0·8)	0·03 (0·03)	0·05 (0·18)	ND	ND
Triethoxy sulphate		>1·0 (WS)	0·2–0·5 (1·5)	0·05 (0·2)	0·02 (0·06)	0·03 (0·5)	ND
Hexaethoxylate		ND	ND	0·02–0·05 (0·035)	ND	0·01 (0·07)	ND

ND = Not determined WS = Water soluble IS = Insoluble

*Values of critical micelle concentration (mM) are given in parentheses.

†No histamine release observed at given concentration; higher levels not examined.

‡Surfactant insoluble at 22°C.

As shown in Table 1, one animal showed a spontaneous release of 29·4% of the total cellular histamine in the presence of buffer only, but this was exceptional.

It was never possible to release all of the mast-cell histamine by surfactants. In preliminary experiments, compound 48/80 was found to release only 83% of the total histamine at a concentration of 1 μg/ml or above, and at lower concentrations of compound 48/80 the release of histamine was proportionately less. Bloom & Haegermark (1965) showed that with compound 48/80 a concentration of 0·5 μg/ml was sufficient to release 78% of total mast-cell histamine. Röhlich *et al.* (1971) reported that high protein concentrations altered the amount of compound 48/80 required to degranulate mast cells *in vitro*, and so in initial experiments with surfactants serum was also added (Fig. 1). As it was obvious that its presence necessitated higher concentrations of surfactant to cause maximum histamine release, serum was omitted in subsequent experiments. Putnam (1948) described the strong affinity of surfactants to protein.

While there are numerous reports of the detailed mechanism of histamine release from rat mast cells *in vitro* by a variety of agents, such as compound 48/80, antigens and drugs, the mode of action of surface-active compounds has been covered less adequately. Meng & Westerholm (1963) suggested that certain emulsifiers disrupted mast cells and released histamine by virtue of their surface activity, and pointed out that specific metabolic inhibitors which prevented the enzymatic process of degranulation by compound 48/80 did not similarly alter the disruptive effects of these emulsifiers. Bloom & Haegermark (1965), Lagunoff (1972) and Röhlich *et al.* (1971) have all shown that, following the degranulation of mast cells by compound 48/80 or polymyxin B, the structural integrity of mast cells and their intracellular organelles is preserved. This was not seen with surfactants, however. The electron micrograph of mast cells exposed to sodium lauryl sulphate (Fig. 2) shows obvious signs of physical disruption of the cells, similar to the effects of hypotonicity (Bloom & Haegermark, 1967; Mann, 1969) or decylamine, which is also surface active (Bloom & Haegermark, 1967). That other cellular constituents of the peritoneal washings were not similarly affected indicates a particular susceptibility of rat mast cells to surfactants, as has been shown for hypotonicity by Mann (1969).

Thus, the cytolytic action of various surfactants upon mast cells may be ascribed to their specific interaction with membranes. Various types of surfactant produced maximum histamine release at different concentrations, suggesting a dependence upon the precise chemical nature not only of the polar head groups but also of the alkyl lipophilic chain. In particular, the chains of intermediate length (C_{12} and C_{14}) showed the greatest cytolytic activity. Schott (1973) has suggested that in any series of surfactants, those of intermediate lipophilic chain length (e.g. C_{10}–C_{14}) possess the greatest haemolytic effectiveness because of a combination of two mutually opposing factors. The oil–water partition coefficient (which is taken as representative of the dissolution of a surfactant in the apolar membranes of a cell and thus may cause lysis) will increase with increasing chain length, but the monomer concentration of a surfactant (i.e. the number of free molecules in solution below the CMC) will decrease with increasing chain length. Thus, a maximum concentration of surfactant in the lipophilic environment of the cell membrane will occur at intermediate chain length—C_{12} for alkyl sulphates and C_{11} for alkyl carboxylates. The data in Table 2 approximate to this.

In relation to this point, Rosen (1972) has reviewed how intrinsic properties of surfactants (such as surface-tension reduction, micelle formation, wetting, foaming and detergency) are dependent upon the hydrophobic and hydrophilic portions of the molecules, and changes in chain length, nature of head group and presence or absence of ionic charge greatly affect these properties. As shown in Table 2, the uncharged alkyl triethoxylates were generally as effective as the corresponding alkyl sulphates in releasing histamine, but it is probable that their mode of action upon the mast-cell membranes differs from that of anionic surfactants. Pethica & Schulman (1953) described different types of lysis of red blood cells depending upon the type of surfactant used: non-ionic surfactants (e.g. ethoxylated alcohols), by virtue of their surface pressure in solution, would exceed the collapse pressure of the liproproteins of the surface membrane of the cell, whereas ionic detergents would cause increased interfacial pressures due to ion–ion interactions during penetration through the cell membranes.

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THE EFFECT OF SURFACTANTS UPON MAMMALIAN CELLS *IN VITRO*

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Abstract—A series of highly pure surfactants were examined *in vitro* for their capacity to inhibit DNA synthesis in guinea-pig kidney fibroblasts, to release histamine from isolated rat peritoneal mast cells, and to effect the cytolytic release of cytoplasmic proteins from guinea-pig kidney fibroblasts. The surfactants studied included a homologous series of carboxylic acid sodium soaps of chain length C_8–C_{16} and a series of chain length C_{12} with different chemical head groups. Of the carboxylic acid soaps, sodium laurate (chain length C_{12}) was more potent at inhibiting DNA synthesis, at releasing histamine and at lysing fibroblasts than either the higher or lower homologues. Exchange of the carboxylic acid head group on the alkyl chain for a sulphate or isethionate moiety increased the potency of the surfactant and introducing three or six ethoxy groups further increased the potency to living cells.

INTRODUCTION

Many laboratory approaches have been reported for examining the irritant effects of applying surface-active compounds (surfactants), such as soaps and detergents, to mammalian skin. At a physico-chemical level, the effects of various surfactants on water-binding capacity (Middleton, 1969), skin permeability (Bettley, 1963), denaturation of the proteins of the stratum corneum (Harrold, 1959) or conductance (Dugard & Scheuplein, 1973) have been studied. More recently, at a biochemical level, the effect of some surfactants on DNA and lipid metabolism (Prottey & Hartop, 1973), epidermal phospholipid metabolism (Mezei, 1975) and acid phosphatase (Rutherford & Pawlowski, 1974) have been reported. The response to surfactants at a cellular level has been studied by Pethica & Schulman (1953), who described their haemolytic properties, by Adams, Painter & Payne (1963), who described the effect of sodium caprylate on yeast cells, and by Bettley (1968), who studied the toxicity of soaps and detergents on yeast cells, erythrocytes and cells of the buccal mucosa. It is questionable, however, whether any of these methods are fully representative of the total possible interactions of surfactants with the skin, as may be observed by clinical assessment following direct application (Weil & Scala, 1971).

In this study, synthetic surfactants of varying length of alkyl chain and polarity of head group were subjected to three different cytological procedures for the examination of certain levels of interaction of surfactants with living cells. At a metabolic level, their effects on DNA metabolism have been studied in guinea-pig kidney fibroblasts using [6-^{3}H]thymidine uptake. Secondly, the release of histamine from rat peritoneal mast cells by surfactants has been studied in an attempt to represent an initial aspect of the dermal inflammatory response, namely the release of histamine from dermal mast cells. Thirdly, the potential cytolytic effects of these surfactants have been studied by measuring the release of radioactively-labelled cytoplasmic proteins from guinea-pig kidney fibroblasts. The effects of the individual surfactants in these procedures have been related to their chemical structure.

EXPERIMENTAL

Materials. Trypsin, from hog pancreas, was obtained from Koch-Light Laboratories Ltd., Colnbrook, Bucks. Eagle's Medium M.E.M. and Medium 199 were obtained as concentrates ($\times 10$) from Wellcome Reagents Ltd., Beckenham, Kent. Falcon culture flasks and tubes were obtained from Biocult, Ayr, Scotland, and [6-^{3}H]thymidine and sodium [^{51}Cr]chromate from the Radiochemical Centre, Amersham, Bucks. [6-^{3}H]Thymidine was diluted with sterile water to give 10 μCi/0·1 ml (5·0 Ci/mmol); sodium [^{51}Cr]chromate was diluted with sterile water to give 100 μCi/0·1 ml (167 mCi/mg Cr). All pure surfactants used in this study were either purchased or synthesized in this laboratory; further details are given in the preceding paper (Prottey & Ferguson, 1976).

Measurement of DNA synthesis in guinea-pig kidney fibroblasts in vitro. Guinea-pig fibroblasts, prepared by trypsinization of guinea-pig kidneys (Ferguson & Prottey, 1974), were cultured in Eagle's medium (Eagle, 1959) at 37°C and were grown to confluency in plastics Falcon flasks. Culture tubes containing graded concentrations of the surfactants were inoculated with a known concentration of fibroblasts, generally about 2×10^5 cells/tube. The concentrations examined were 0·001, 0·005, 0·01, 0·05, 0·1, 0·5, 1·0, 5·0 and 10·0 mM for all three cytological assays. To each tube was added 10 μCi [6-^{3}H]thymidine (equivalent to 2 nmol), the total volume was adjusted to 1·0 ml and the cells were incubated overnight at 37°C. The cells were collected by centrifugation and re-suspended in medium fortified with 0·2 mmol unlabelled thymidine (100 times the concentration of radioactive thymidine used) to wash out residual radioactive thymidine. After centrifugation, the medium was removed and the cell pellets were extracted three times with 1 ml ice-cold 5% (w/v)

trichloroacetic acid, followed by a single extraction with 1 ml absolute ethanol. These extracts were discarded. The cell pellet residues were finally extracted three times with 1 ml 5% (w/v) trichloroacetic acid at 70°C to solubilize the labelled DNA. Aliquots of the combined hot trichloroacetic acid extracts were then counted in a Packard 4000 Liquid Scintillation Counter (with efficiencies for tritium of about 8%) to determine the level of thymidine uptake into DNA.

Measurement of histamine release from rat peritoneal mast cells in vitro. Rat peritoneal mast cells were harvested by the method of Perelmutter & Khera (1970) immediately prior to incubation with surfactant. The rats were killed by carbon-dioxide asphyxiation and the peritoneal cavity was irrigated with 30 ml of Medium 199 (Morgan, Morton & Parker, 1950) containing 0·5 mg ethylenediaminetetraacetic acid/ml to reduce cell aggregation. The cells were collected by gentle centrifugation (10 min at 250 **g**) and were re-suspended in a buffered salt solution (154 mM-NaCl, 2·7 mM-KCl and 0·9 mM-$CaCl_2$) containing 10% v/v Sörensen's buffer (67 mM-Na_2HPO_4 + KH_2PO_4, pH 6·8). The mast cells were incubated for 3 hr at 37°C with a range of concentrations of each surfactant (from 0·001 to 10·0 mM). The cell density was generally about 1×10^5 cells/assay tube. After incubation with the surfactants, the cells were centrifuged and the separated supernatant solution and cell pellet were assayed for histamine by the method described by von Redlich & Glick (1965) using a Locarte Spectrofluorimeter. For each surfactant the concentration was determined at which all the available histamine was released. There was always a background loss of histamine of about 10% of the total due to handling. As the maximum amount of histamine released was never more than 90% of the total histamine content of the cells, results obtained for histamine release in the presence of surfactants were expressed as a percentage of the histamine available for release rather than as a percentage of the total.

Measurement of the release of covalently bound chromium from guinea-pig kidney fibroblasts in vitro. Three days before an experiment, confluent guinea-pig fibroblast cultures were trypsinized and seeded into a series of plastics Falcon tubes at known concentrations of about 2×10^5 cells/ml and then left at 37°C to attach to the vessel walls and to continue to replicate. All subsequent operations were carried out upon cells in these Falcon tubes. Fibroblasts, 3 days after seeding into assay tubes, were pulsed with 100 μCi sodium [^{51}Cr]chromate for 3 hr, after which time the cells were washed by decantation with 4×1 ml portions of phosphate-buffered saline (see above), and the washings were discarded. The cells were left for 24 hr in Eagle's medium containing 20% guinea-pig serum at 37°C to remove non-covalently bound ^{51}Cr, and were then incubated for 3 hr at 37°C in 1 ml medium without serum but containing varying concentrations of the surfactants (from 0·001 to 10·0 mM). Serum was deliberately omitted from the medium as certain surfactants bind tightly to serum proteins, thereby reducing their effective concentrations. After 3 hr the cells were centrifuged at 250 **g** for 10 min, because some surfactants caused the cells to detach from the vessel wall during incubation. The degree of lysis was determined by withdrawing 0·5 ml supernatant solution and counting the radioactivity using a Packard 5000 Auto Gamma Spectrometer. To the remainder in the tubes, 0·5 ml ethanolamine was added and the tubes and contents were incubated for 48 hr to dissolve the cells completely. This aliquot was then counted to determine the level of radioactivity remaining in the cells (allowance being made for the 0·5 ml supernatant also remaining with the cells). There was always a background loss into the medium of about 10% of the total radioactivity incorporated into the cells as some cells were unavoidably damaged during handling. This background loss was measured for each assay. Also the maximum amount of cytoplasmic radioactivity available for release by cell lysis, determined by freezing and thawing a portion of the cells three times, was about 80% of the total radioactivity incorporated into the cells. Hence, the cytolytic effect was recorded in terms of the proportion of radioactivity available for lytic release over and above the background loss, rather than as a percentage of the total radioactivity initially incorporated into the cells.

RESULTS

The effect of surfactants upon DNA synthesis in guinea-pig kidney fibroblasts in vitro

For each surfactant a range of concentrations was examined to determine the concentration required to produce a 50% reduction in [6-^{3}H]thymidine incorporation into DNA. Table 1 shows that for the carboxylic acid soaps, sodium laurate (chain length C_{12}) was more potent than either the higher or lower homologues. Replacing the carboxylic acid head group on the lauryl chain by a sulphate, isethionate or monoethoxylate moiety did not cause any marked change in the concentration required for 50% inhibition of DNA synthesis. However, the introduction of three or six ethoxy groups increased the potency of the surfactant to affect DNA synthesis, 50% inhibition occurring at a much lower concentration.

Effect of surfactants on the release of histamine from rat peritoneal mast cells in vitro

As histamine release was found to occur over a narrow concentration range, it was not possible to determine the concentration that released 50% of the available histamine, so for each surfactant the concentration determined was that at which all the available histamine was released. For the homologous series of soaps, sodium laurate was the most potent (Table 1). Increasing the polarity of the head-group, by substituting an isethionate or a sulphate moiety for the carboxyl group, increased the potency of the C_{12} surfactant, a lower concentration being required for histamine release.

With ethoxylated surfactants, the effectiveness of the monoethoxy group did not differ significantly from that of sodium laurate, but increasing the degree of ethoxylation to three or to six ethoxy groups increased the effectiveness greatly, in that histamine was released by a tenth of the concentration required in the case of sodium laurate.

Table 1. *Effect of surfactants* in vitro *on DNA synthesis in guinea-pig fibroblasts, on histamine release from rat peritoneal mast cells and on lysis of guinea-pig fibroblasts*

Surfactant	Structure	Concn (mM) of surfactant required to		
		Inhibit DNA synthesis by 50%*	Release all available histamine†	Release 50% of available ^{51}Cr*
Sodium caprylate	C8:0	0·6	>5·0	>10·0
caprate	C10:0	0·4	1·0	>10·0
laurate	C12:0	0·1	0·4	7·1
myristate	C14:0	0·2	0·5	7·7
palmitate	C16:0	0·3	1·0	>10·0
Sodium lauryl sulphate	$CH_3 \cdot [CH_2]_{10} \cdot CH_2 \cdot SO_4Na$	0·06	0·03	0·22
lauroyl isethionate	$CH_3 \cdot [CH_2]_{10} \cdot COO \cdot [CH_2]_2 \cdot SO_3Na$	0·08	0·15	0·25
Lauryl monoethoxylate	$CH_3 \cdot [CH_2]_{10} \cdot CH_2 \cdot OCH_2 \cdot CH_2OH$	0·06	0·46	0·21
triethoxylate	$CH_3 \cdot [CH_2]_{10} \cdot CH_2 \cdot [OCH_2 \cdot CH_2]_3 \cdot OH$	0·01	0·03	0·30
hexaethoxylate	$CH_3 \cdot [CH_2]_{10} \cdot CH_2 \cdot [OCH_2 \cdot CH_2]_6 \cdot OH$	0·01	0·03	0·02

*In guinea-pig fibroblasts.
†In rat peritoneal mast cells.
In each experiment, these surfactants were examined in batches. Sodium lauryl sulphate was included in each batch and the means of five estimations were 0·06 ± 0·003, 0·03 ± 0·004 and 0·22 ± 0·005 mM for the studies involving inhibition of DNA synthesis, release of histamine and release of ^{51}Cr, respectively.

The cytolytic effect of surfactants upon guinea-pig kidney fibroblasts in vitro

The cytolytic effect of this series of surfactants was investigated at various concentrations by measuring the release of cytoplasmic proteins labelled with covalently bound ^{51}Cr from fibroblasts. The results are recorded in Table 1 as the concentration of surfactant required to release 50% of the available chromium. Sodium laurate (C_{12}) and sodium myristate (C_{14}) were the only soaps to produce a cytolytic effect at concentrations below 10 mM. Exchanging the carboxylate group for a sulphate, isethionate or mono- or triethoxy group produced a dramatic effect; cell lysis was produced at concentrations of 0·2–0·3 mM. Moreover, introducing six ethoxy groups further increased this effect, as a concentration of only 0·02 mM produced cell lysis.

DISCUSSION

Three distinct cytological models were chosen to represent various types of interaction between surfactants and skin cells. Firstly, DNA metabolism was chosen as it is known to be stimulated in the rat epidermis following surfactant application to the skin (Prottey & Hartop, 1973). Basal cells could not be used as they have limited viability in culture and do not lend themselves well to successive subculturing (Prottey, Tovell & Ferguson, 1975). Instead fibroblasts were taken to be representative of normal diploid cells. DNA synthesis was chosen as a parameter for cellular viability because it is a metabolic process that requires an intact cellular structure and because cells will invariably duplicate their nuclear DNA during the S-phase of the cell cycle immediately preceding mitosis. Measurement of DNA synthesis by incorporation of [6-^{3}H]thymidine is easy and rapid compared with counting mitotic figures or cell numbers. Preliminary studies (Ferguson & Prottey, 1974) were made to ensure that optimum conditions for DNA labelling were chosen. Microscopic examination showed that the cells were not lysed at the surfactant concentration required to cause 50% inhibition of DNA synthesis.

Mast cells *in vitro* were chosen as they are known to be involved in the inflammatory response elicited by the cutaneous application of primary irritants (Steele & Wilhelm, 1970). A separate study (Prottey & Ferguson, 1976) showed that in a mixed population of rat peritoneal cells exposed to various surfactants, only mast cells were disrupted, while other cells such as macrophages, lymphocytes and granulocytes were resistant to damage. A similar finding was reported by Mann (1969) for the effect of hypotonicity on rat peritoneal mast cells. These observations suggest a particular fragility of mast cells *in vitro* to certain compounds.

In order to measure lysis of fibroblasts, a cytoplasmic marker was required that would be incorporated into cells without affecting their normal behaviour in culture, that would be bound firmly within the cells until lysis occurred and then be released but not subsequently re-utilized by other intact cells, and that could be determined by a simple method both within intact cells and after lysis. Sodium [^{51}Cr]chromate satisfied these criteria, having a suitable half-life (28 days) and being a fairly strong γ-emitter. In the hexavalent form, this nuclide is incorporated into cells and is reduced to the trivalent form upon attachment to lysine or arginine units of cytoplasmic proteins (Gray & Sterling, 1950). When a cell is lysed, ^{51}Cr-labelled proteins leak out. As the trivalent ^{51}Cr cannot be re-utilized by other cells (Ebaugh, Emerson & Ross, 1953), measurement of ^{51}Cr radioactivity in the medium gives a measure of cell lysis.

In each of the three methods described, the same group of surfactants was used, and two points may be made from the data in Table 1. Firstly, the concentration of surfactant required to inhibit DNA synthesis was always lower than that required to release histamine from mast cells, which was in turn lower than the concentration required for fibroblast lysis. For example, using sodium laurate, 0·1 mM caused 50% inhibition of DNA metabolism and 0·4 mM

released histamine from mast cells, but 7·1 mM was required to produce 50% cytolysis of fibroblasts. Secondly, the ranking of individual surfactants in terms of their potency in any particular assay procedure was uniform and depended upon the chemical structure of the surfactants. We have observed consistently the trend that soaps of C_{12} chain length are more potent in their action than higher or lower homologues. The tri- and hexaethoxylates were effective at the lowest concentrations with the exception of the triethoxylate in the fibroblast assay, and these were followed by sodium lauryl sulphate, sodium lauroyl isethionate and the carboxylates in the order laurate, myristate and caprate.

Although similar reports of maximum intensity for the laurate anion in various other investigations of skin irritancy have been described (Choman, 1963; Dugard & Scheuplein, 1973; Emery & Edwards, 1940; Howes, 1975), it must not be assumed that the present procedures are directly representative of the complex phenomenon of skin irritation. Other primary non-allergic irritants, as well as surfactants, have a direct action on the skin, invoking cytolysis, altered metabolism and release of pharmacological mediators. For example, hexadecane has been studied by Cowan & Mann (1971), acids and alkalis by Nagao, Strond, Hamada, Pinkus & Birmingham (1972) and solvents by Steele & Wilhelm (1970). However, this does not necessarily imply direct action of the irritants with all of the cells concerned. DNA synthesis may be mediated by chalones or cyclic AMP (Duell, Kelsey and Voorhees, 1975), which themselves have been altered by the irritant. Primary irritants may not affect mast cells directly and cause histamine release *in vivo*; rather there may be a prior fixation of complement, leading to the formation of specific histamine-releasing polypeptides (Ruddy, 1974). Moreover cytolysis may be a consequence of lysosomal enzymes from the epidermis or the leucocytes (Goldstein, 1974) rather than a direct action of the irritant at the plasma membrane of the cell in question.

However, the close relationship apparent in the data given here and by others for different aspects of skin inflammation suggests a value in this approach to determining surfactant toxicity, even though the mechanism of the initial mediation of the primary irritation phenomenon has yet to be elucidated.

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SHORT PAPERS

SHORT-TERM TOXICITY OF CHOCOLATE BROWN FB IN PIGS

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Summary—Chocolate Brown FB was mixed with diet and fed to groups of three male and three female pigs for 16 wk in doses of 0 (control), 25, 250 or 1000 mg/kg/day. There were no significant differences between the control and treated groups in body-weight gain, haematological examinations, urine analyses or organ weights. The only effect attributable to treatment was a pigmentation of lymphoid tissue. Additional groups of pigs were given 0 (control) or 10 mg/kg/day for 90 days and no pigment was found. The no-untoward-effect level established in this study was therefore 10 mg/kg/day.

Introduction

Chocolate Brown FB is listed in the 1971 Colour Index, but has no number or specification assigned to it. It is prepared by diazotizing naphthionic acid and coupling the product with osage orange extract, which is principally a mixture of morin (2',3,4',5,7-pentahydroxyflavone; C.I. (1971) no. 75660) and maclurin (pentahydroxybenzophenone; C.I. (1971) no. 75240).

Given ip, Chocolate Brown FB had LD_{50} values of 200 mg/kg in mice and 250–500 mg/kg in rats (Gaunt, Hall, Farmer & Fairweather, 1967), but mice and rats tolerated daily oral doses of 1 and 2 g/kg, respectively, for 3 wk without any toxic manifestations. In a short-term feeding study in rats, the same authors obtained a no-effect level of 150 mg/kg/day. In a long-term feeding study in rats, no carcinogenic potential was detected by Gaunt, Brantom, Grasso, Creasey & Gangolli (1972) and the no-untoward-effect level was approximately 50 mg/kg/day. Similarly Gaunt, Brantom, Grasso & Kiss (1973) detected no carcinogenic effects in mice given the material in the diet at levels of 10,000 ppm for 80 wk and the no-untoward-effect level was 3000 ppm, which was equivalent to an intake of approximately 430 mg/kg/day. The main finding in all these studies was the presence of pigment in the cells of the reticulo-endothelial system.

The present study formed the final part of an assessment of the safety-in-use of Chocolate Brown FB, carried out as part of the BIBRA safety evaluation programme in the light of reports of the Food Standards Committee (1964) and the Joint FAO/WHO Expert Committee on Food Additives (1965) stressing the need for further evidence of safety-in-use if Chocolate Brown FB were to continue to be used in food. The colouring is included in the current UK list of permitted food colourings (The Colouring Matter in Food Regulations 1973, Statutory Instrument 1973 no. 1340), but is apparently no longer in use, primarily because the raw materials are not available. This technological problem is exacerbated by the lack of a clear specification for Chocolate Brown FB and, in a recent report, a committee of the Commission of the European Communities (1975) expressed its inability to relate available biological data to the material used in food. The committee considered the colouring to be unacceptable for food use, a decision likely to be endorsed by the UK Food Additives and Contaminants Committee in its forthcoming report.

Experimental

Materials. The sample of Chocolate Brown FB used in this study was supplied through the Food Colours Committee of the Chemical Industries Association. There is no British Standard specification for Chocolate Brown FB, but this sample was stated to contain 81·8% pure dye, 6·0% volatile matter (loss at 135°C), 0·05% water-soluble material, 0·9% ether-extractable material, 11·3% chlorides and sulphates (expressed as sodium salts), 0·5 ppm arsenic, 2·0 ppm lead and 14·0 ppm copper.

Animals and diet. Three 9-wk-old litters each of four male and four female pigs of the Large White strain, were purchased from a minimal-disease herd. The pigs (weighing initially approximately 18 kg) were caged individually and fed Hi-lean Pig Rearer Pencils (British Oil and Cake Mills Ltd., London) at a rate of 0·5–1.5 lb/day, and water was provided *ad lib.*

Experimental design and conduct. The pigs were allocated to four groups of three males and three females so that each group consisted of one male and one female from each litter. These groups were given 0 (control), 25, 250 or 1000 mg Chocolate Brown FB/kg body weight/day for 16 wk. The colouring was mixed with diet to provide concentrations of 0·5, 5 and 20% and the mixture was diluted with an equal weight of Golden Syrup (Tate & Lyle Ltd., London). Each pig was given the appropriate mixture in an amount equivalent to 10 g/kg body weight before the main feed. These mixtures were eaten immediately

and the procedure ensured that the animals received the whole dose with the minimum of spillage. The control pigs had a similar treatment routine but received no colouring. The animals were weighed initially and then twice weekly.

For haematological examinations, blood samples were collected from an ear vein of each pig at 6 wk and from the superior vena cava immediately prior to autopsy at wk 16. Measurements were made of the haemoglobin concentration and packed cell volume, together with counts of erythrocytes, reticulocytes and total and differential leucocytes. During wk 6 and 13, the drinking-water was removed and the urine was collected for a period of 1 hr. The urine was examined for the presence of glucose, ketones, bile salts and blood and a study of the constituents of the sediment obtained by centrifuging included a count of the number of cells.

The pigs were killed by exsanguination after iv administration of sodium pentobarbitone. At autospy the organs were examined for macroscopic abnormalities and the brain, heart, lungs, liver, spleen, kidneys, adrenals, gonads, pituitary, thyroid, stomach, small intestine and caecum were weighed. Samples of these organs and of the salivary glands, lymph nodes, pancreas, urinary bladder, ureters, urethra, aortic arch, trachea, gall bladder, oesophagus, colon, rectum, skeletal muscle, sternum, diaphragm, tongue, and seminal vesicles, vas efferens, vas deferens and prostate or uterus, Fallopian tubes and vagina were preserved in 10% buffered formalin. Paraffin-wax sections of these tissues were stained with haematoxylin and eosin for histopathological examination. In an additional study, groups of three male and three female pigs were given 0 (control) or 10 mg Chocolate Brown FB/kg/day for 90 days and the same tissues were examined histopathologically.

Results

No abnormalities of appearance or behaviour were observed in any of the pigs. The faeces of all the treated animals were dark brown throughout the study and the urine of the pigs receiving the highest levels of the colouring developed a light- to medium-brown coloration as the study progressed. There was an indication of a reduction in the body-weight gain of the male pigs given the top dietary level of Chocolate Brown FB, the mean gain by wk 15 being 17·5 kg in this group compared with 20·5 kg in the male control group, but this effect was not statistically significant. No statistically significant differences between the control and test animals were detected in the haematological study (Table 1). No abnormal constituents were detected in the urines from the control or treated pigs and the numbers of cells in the urine were similar in all groups. The only statistically significant ($P < 0·01$) change in the organ weights was in the weight of the lungs of the males given 1000 mg Chocolate Brown FB/kg/day. When this value was expressed relative to the body weight of the animals, it remained below the control value but the difference was not statistically significant (Table 2).

At autopsy, one animal given 1000 mg Chocolate Brown FB/kg/day was found to have an ovarian cyst, which was shown histologically to be a benign cystadenoma. The only consistent macroscopic finding was a brown coloration of the lymph nodes of all the treated animals. On histopathological examination, the pigmentation was detectable in the pigs given the highest level of treatment and in one of the females given 250 mg/kg/day. Traces of pigment were also detected in some of the macrophages in the lymph nodes of two of the male pigs given 25 mg/kg/day. In the additional group of three male and three female pigs given 10 mg Chocolate Brown FB/kg/day, no pigment was seen in the histopathological examination, although the lymph nodes were seen at autopsy to be pale brown in colour.

Some pigmentation was detected histologically in the mucosa of the alimentary tract, and in the lymphoid collections within it, in the pigs given the highest level of Chocolate Brown FB. The kidneys of one male pig receiving 1000 mg/kg/day were dark brown

Table 1. *Haematological findings in pigs fed Chocolate Brown FB in doses of 0–1000 mg/kg/day for 16 wk*

Sex and dose level (mg/kg/day)	Hb (g/100 ml)	PCV (%)	RBC (10^6/mm^3)	Retics (% of RBC)	Leucocytes: Total (10^3/mm^3)	Leucocytes: Differential (%) N	E	L	M
Male									
0	12·9	45	6·85	0·48	14·7	24	2	72	2
25	13·3	45	6·62	0·47	13·0	23	2	73	2
250	12·6	43	6·31	0·37	12·4	20	2	77	1
1000	13·5	47	6·38	0·23	15·4	23	2	74	1
Female									
0	13·4	44	7·09	0·43	13·8	29	2	67	2
25	12·5	39	6·23	0·36	9·8	27	1	71	1
250	13·4	42	6·86	0·76	12·1	13	1	85	1
1000	13·1	43	6·33	0·63	13·2	34	2	62	2

Hb = Haemoglobin PCV = Packed cell volume RBC = Red blood cells Retics = Reticulocytes
N = Neutrophils E = Eosinophils L = Lymphocytes M = Monocytes

Figures are means for groups of three pigs and those for the treated animals do not differ significantly from those of the controls at $P = 0·05$ (Lord (1947) range test). In similar studies at wk 6, there were again no significant differences between control values and those for treated animals.

Basophils did not account for more than 0·5% of the leucocytes and no inclusions were seen in the erythrocytes.

Table 2. *Relative organ weights of pigs fed Chocolate Brown FB in doses of 0–1000 mg/kg/day for 16 wk*

Sex and dose level (mg/kg/day)	Relative organ weights (g/kg body weight)													Terminal body weight (kg)
	Lungs	Brain	Heart	Liver	Spleen	Kidney	Stomach	Small intestine	Caecum	Adrenals	Gonads	Pituitary†	Thyroid	
Male														
0	7·18	2·52	4·15	18·68	1·29	2·60	9·02	23·55	1·78	0·553	2·88	3·70	0·086	41·0
25	5·83	2·28	3·60	18·55	1·07	2·28	9·35	23·65	1·51	0·493	3·43	3·60(2)	0·075(2)	43·0
250	6·51	2·58	3·89	20·66	1·29	2·67	9·75	24·81	1·88	0·559	2·77	3·75(2)	0·097	40·0
1000	5·95	2·61	3·96	17·36	1·15	2·77	10·21	26·01	2·12	0·586	2·79	3·68(1)	0·089	38·0
Female														
0	6·28	2·23	3·72	17·66	1·16	2·30	10·27	24·46	1·99	0·597	0·086	4·33	0·084	42·0
25	5·94	2·34	3·98	18·10	1·04	2·56	9·16	26·89	1·94	0·587	0·079	4·94	0·083	40·8
250	6·13	2·28	3·81	15·16	1·16	2·35	10·02	23·64(2)	2·00(2)	0·540	0·088	3·90(1)	0·078	43·0
1000	6·15	2·22	3·76	14·91	1·08	2·56	9·26	24·15(2)	2·42	0·621	0·078	3·88	0·069	39·8

†Relative weights of the pituitary gland are expressed in mg/kg body weight.

Values are means for groups of three pigs, except where the group size is indicated in parenthesis. No values for the treated groups differed significantly (Lord (1947) range test) from the corresponding control value.

and pigmentation of the convoluted tubules was seen on histological examination. No other findings were seen at autopsy or on histopathological examination of the tissues.

Discussion

In this study, the only measurement that differed from the control value to a statistically significant degree was a decrease in the weights of the lungs of the males given 1000 mg Chocolate Brown FB/kg/day. No similar effect was apparent in the female pigs or in the values for the male animals when these were expressed relative to body weight. This was considered, therefore, to be a chance finding of no toxicological significance. Variations that occurred in the weights of other organs, including the pituitary, were not statistically significant and could be attributed to the small numbers of observations.

The cystadenoma of the ovary found in one pig given the highest dose level was benign. Again, in view of the small numbers of pigs used, no definite conclusion could be drawn about its significance. In long-term studies in both rats (Gaunt *et al.* 1972) and mice (Gaunt *et al.* 1973), there was no evidence of any increase in ovarian lesions despite treatment over the greater part of the life-span of the animals.

The main finding in this study in pigs, as in those in mice (Gaunt *et al.* 1973) and rats (Gaunt *et al.* 1967 & 1972), was the presence of pigment in the cells of the alimentary canal, in the lymph nodes and in the renal tubules. However, in this study, no pigment was found in the Kupffer cells of the liver, as was observed in the previous studies in mice and rats. The pigment seen in the rat and mouse was shown not to be lipofuscin, and could have been the colouring itself or a metabolite, possibly bound to protein. Since the colouring is readily water-soluble, it is unlikely that the pigment represents the free colouring, as this would be removed during processing for histological examination. Gangolli (1969) showed that the protein-binding ability of this colouring (0·01 g colouring/100 g protein) was the lowest in a series of azo food colourings. Thus it is unlikely that it is the colouring itself, either free or bound to protein, that is taken up. Fore, Walker & Golberg (1967) have shown that Chocolate Brown FB can be degraded *in vitro* by rat-liver homogenates. It is likely, by analogy with other azo colourings (Walker, 1970), that metabolism occurs in the gastro-intestinal tract. Gaunt *et al.* (1973) considered that the materials taken up by the reticulo-endothelial system were probably metabolites of the colouring bound to protein. In the present study in pigs, as in the previous short- and long-term studies in rodents (Gaunt *et al.* 1967, 1972 & 1973), the pigmentation was not associated with any change in organ weights or with tissue damage.

This study established a no-untoward-effect level in pigs of 10 mg/kg/day. However, in view of the doubtful toxicological significance of the findings at other levels, including the presence of pigment, it is likely that the true no-untoward-effect level is much higher than this.

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METABOLISM OF OCHRATOXINS A AND B IN THE PIG DURING EARLY PREGNANCY AND THE ACCUMULATION IN BODY TISSUES OF OCHRATOXIN A ONLY

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Summary—A mixture of ochratoxins A and B (0·38 and 0·13 mg/kg body weight) was fed to pigs daily for 8 days during early pregnancy. Compared with ochratoxin A, its dechloro-derivative ochratoxin B was poorly absorbed and preferentially hydrolysed in the intestinal tract. As a result, ochratoxin A was excreted in the faeces as the unchange toxin and its hydrolysis product ochratoxin α, but ingested ochratoxin B was completely hydrolysed to ochratoxin β. Ochratoxin A and the two metabolites, ochratoxins α and β, were also excreted in the urine, but while the latter metabolite was probably formed from ochratoxin B in the liver, the former may have been absorbed from the intestine. Neither toxin crossed the placenta to the foetus and because ochratoxin B was preferentially metabolized, only ochratoxin A accumulated in the muscle, liver and kidneys of the dam.

Introduction

In an experiment designed to test the effects of feeding mixed ochratoxins to pigs in early pregnancy (21–28 days of gestation) it was found that although ochratoxin A accumulated in the maternal tissues, including the placenta, it did not reach the foetus (Shreeve, Patterson, Pepin, Roberts & Wrathall, 1976). This was consistent with the apparent lack of any teratogenic effect when ochratoxins A and B in oral doses of 0·38 and 0·13 mg/kg body weight, respectively, were administered together daily for 8 days at a stage of pregnancy when the rapidly developing foetus was most vulnerable.

This communication reports data relating to the metabolism of ochratoxins A and B in the pig.

Experimental

Animal experiment. The conduct of this experiment has been described in detail elsewhere (Shreeve *et al.* 1976) but, briefly, two pregnant 8–10-month-old gilts were fed a diet containing a crude ochratoxin extract from day 21 to day 28 of gestation, inclusive, and were slaughtered on day 29 or 30, when the foetuses were examined and tissues were taken for analysis. The daily oral dose was administered in 0·5 kg sow and weaner meal and was equivalent to 0·38 mg ochratoxin A and 0·13 mg ochratoxin B/kg body weight in each case.

Crude ochratoxin extract. Cultures of *Aspergillus ochraceus* (NRRL 3519 and 5175) grown on cereal substrates, as described by Shreeve *et al.* (1976), were homogenized in water using an 'Atomix' blender. Before extraction with chloroform, the pH of the homogenate was adjusted to 2·0 by the addition of 2·0 N-HCl. Extracts were checked for the presence of ochratoxins A and B using TLC, and were then bulked and concentrated to a convenient volume using a rotary evaporator. The final concentrations of toxins in the extract were 0·19 mg ochratoxin A and 0·06 mg ochratoxin B/ml.

Standard and purified toxins. Pure ochratoxin A, purchased from Calbiochem Ltd., Hereford, was used as the 'fluorescence standard' in the semi-quantitative TLC assay of ochratoxins A and B. Ochratoxin B in a quantity of a few μg was obtained from the crude toxin extract by preparative TLC (see below) and purified by extraction from chloroform into 0·1 M-$NaHCO_3$ solution followed by acidification of the aqueous phase and re-extraction with chloroform. The corresponding dihydro-isocoumarin carboxylic acids, ochratoxins α and β, were prepared by hydrolysing ochratoxins A and B, respectively, according to the method of van der Merwe, Steyn & Fourie (1965) and the preparation of these derivatives was monitored by TLC and ultraviolet spectrophotometry (see below). A preparation of ochratoxin α was used as the 'fluorescence standard' in the semi-quantitative TLC assay of ochratoxins α and β (see below).

Ultraviolet spectrophotometry. Concentrations of the four ochratoxins were determined using published or experimentally determined molar extinction coefficients, ε. Spectra of ethanolic solutions of each toxin preparation were verified using a Unicam SP800 recording spectrophotometer and in each case a spectral shift was observed on the addition of aluminium chloride (0·2 ml saturated solution of $AlCl_3$ in ethanol/5 ml) due to complex formation with the vicinal phenolic hydroxyl and carbonyl groups. The relevant spectrophotometric data are reproduced in Table 1.

Thin-layer chromatography. For most purposes glass plates were layered with 250 μm silica gel GHR (Kiesel gel G-HR, Macherey & Nagel, Düren) but

Table 1. *Spectrophotometric data for four ochratoxins*

Ochratoxin	Observed λ_{max} in ethanol (nm)	ϵ at λ_{max} (reference*)	Bathochromic shift on adding $AlCl_3$ (nm)
A	332	6400 (a)	28
α	338	5600 (b)	14
B	318	6500 (c)	28
β	322	6500 (d)	18

*References: (a) Steyn (1971); (b) van der Merwe *et al.* (1965); (c) Chu, Noh & Chang (1972); (d) experimentally determined on the hydrolysis product of ochratoxin B—spectra reported by Steyn & Holzapfel (1967).

Values for λ_{max} and ϵ are pH dependent. Thus, the addition of ammonia produced a marked bathochromic shift with an increased value for ϵ. Values for ethanolic solutions quoted above differed little on the addition of hydrochloric acid.

500 μm layers were used for preparative work. Ready-spread plates (Merck silica gel 60, E. Merck AG, Darmstadt) were used for semi-quantitative analysis. Standard toxins or extracts were spotted and chromatograms were developed with toluene–ethyl acetate–90% formic acid, 60:30:10, by vol. (Scott, Lawrence & van Walbeek, 1970) by allowing the solvent front to advance 100 mm in lined equilibrated tanks. After the solvent had evaporated at room temperature, ochratoxin A (20 ng or more) could be seen as a spot fluorescing greenish-blue under long-wave ultraviolet light. This intensified to blue-violet after the spraying of the plate with the aluminium chloride spray reagent of Stack & Rodricks (1971). R_F values for the ochratoxins A, B, α and β were 0·44, 0·32, 0·30 and 0·24, respectively, using ready-spread plates. Somewhat higher values were obtained with GHR plates.

In the TLC analysis of tissue and other extracts, pure ochratoxin A was used as the 'fluorescence standard' for ochratoxins A and B and a standardized ochratoxin α preparation was used for ochratoxins α and β. Two qualitatively different 'fluorescence standards' were required for visual matching because the two derivatives fluoresced a much darker blue than the parent toxins, but corrections had to be made for differences in intrinsic fluorescence, the relevant ratios for A:B and α:β being about 3:1 (see below). Concentrations of toxins in various extracts were calculated from the formula:

$$\frac{S.V.F.10^2}{v.W.R}\ \mu g/g \text{ tissue etc.}$$

where S = quantity (ng) of appropriate 'fluorescence standard' equivalent in intensity to unknown spot, v = volume (μl) of extract spotted, V = total volume (ml) of extract, W = weight (g) of tissue etc. extracted, R = experimentally determined recovery (%) of added toxin, and F = fluorescence factor (experimentally determined values for A = 1·0; α = 1·0; B = 3·3; β = 3·1).

Extraction of toxins from tissues, serum and excreta. The method of Krogh, Axelson, Elling, Gyrd-Hansen, Hald, Hyldgaard-Jensen, Larsen, Madsen, Mortensen, Møller, Petersen, Ravnskov, Rostgaard & Aalund (1974) for the extraction of ochratoxin A from tissues was used unchanged in many cases, but slight modifications were made elsewhere in order to maintain a pH of 2–3 during homogenization. Thus, muscle, liver, kidney, placenta and whole foetus were homogenized in 0·01 M-phosphoric acid (H_3PO_4) but 0·1 M-H_3PO_4 was used for serum and urine and 0·2 M-H_3PO_4 for samples of faeces. Extraction with chloroform and column clean-up were effected exactly as described by Krogh *et al.* (1974). Recoveries of added toxins were determined experimentally after adding ochratoxin A to homogenates of muscle, liver and kidney (2.34 ppm), placenta (0·94 ppm), faeces (0·56 ppm), and urine and serum (0·19 ppm), ochratoxin B to serum (0·63 ppm), ochratoxin α to faeces and urine (0·34 ppm), and ochratoxin β to faeces and urine (0·23 ppm). As described above, the values obtained (means of three determinations; see Table 2) were used as correction factors in the estimation of ochratoxins and metabolites in tissues and excreta.

Results and Discussion

As shown in Table 2, ochratoxin A but not ochratoxin B accumulated in maternal tissues and in the placenta. Neither toxin could be detected in the foetus, however, an observation similar to that of Munro, Scott, Moodie & Willes (1973), who administered ochratoxin A (1 mg/kg) iv to pregnant sheep. Both toxins were found in the single sample of serum analysed indicating that ochratoxins A and B were absorbed from the alimentary tract, possibly from the stomach (cf. Galtier, 1974a,b). Ochratoxin A was more readily absorbed than ochratoxin B, the ratio of the concentrations of the two toxins (A:B) in the serum being 4·09 compared with 3·0 in the crude extract fed to the gilts. Ochratoxin A and its metabolite ochratoxin α were present in the urine and in the faeces, but although ochratoxin B was not excreted as such it appeared as its metabolite, ochratoxin β. Compared with the ratio of ingested toxins (A:B = 3·0), the ratio of the concentrations of metabolites α:β in the faeces was 0·71 in one animal and 0·50 in the other, suggesting that ochratoxin B was hydrolysed by intestinal carboxypeptidases more efficiently than ochratoxin A.

Table 2. *Ochratoxin metabolism in pigs given ochratoxins A and B in daily doses of 0·38 and 0·13 mg/kg body weight, respectively, for 8 days during early pregnancy*

	Ochratoxins detected in pig tissues and excreta: levels in												
	Body tissues* (μg/g)					Serum* (μg/ml)		Urine† (μg/mg creatinine)			Faeces‡ (μg/g dry matter)		
	Kidney	Liver	Muscle	Placenta	Foetus								
Animal Ochratoxin...	A	A	A	A	A	A	B	A	α	β	A§	α	β
Expl no. 1	0·70 (0·25)	0·34 (0·19)	0·13	0·06	ND	—	—	0·33	trace	ND	8·1	143·2	201·5
Expl no. 2	0·43 (0·18)	0·30 (0·14)	0·15	0·04	ND	6·99	1·71	0·04	0·18	0·80	1·3	100·4	200·2
Control	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Recoveries of toxin‖ (%)...	47	42	70	75	—	85	62	95	90	62	15	80	58

ND = Not detected

*Tissues taken at slaughter on day 30 of pregnancy. Values are means of three determinations; those in parenthesis are predicted values using regression equations of Krogh *et al.* (1974) and based on calculated equivalent dietary concentrations of ochratoxin A (20·2 and 14·4 ppm for experimental animals nos 1 and 2, respectively).

†Sampled on day 29 on completion of feeding of toxins. On days 25–28, ochratoxin A was detected in four samples taken from experimental animal no. 1 and in five samples from no. 2, in concentrations of 0·04–0·41 μg/mg; creatinine; ochratoxin α was present in trace amounts in one sample from no. 1 and in all five samples (traces–0·28 μg/mg creatinine) from no. 2, and ochratoxin β was detected only in samples from no. 2 (traces–1·26 μg/mg creatinine).

‡Sampled during dosing.

§Because of the low recoveries of this minor constituent, the concentrations quoted are very approximate.

‖Means of three determinations; the levels of the various ochratoxins added are given in Experimental section.

Although traces of metabolites α and β were detected in several urine samples from both animals, the levels were sufficiently high to be quantitated in only two samples obtained from experimental animal 2. The ratio of concentrations α:β was found to be 0·22 in both samples compared with the ratio for A:B of 4·09 in the serum of the same animal. Coupled with the fact that ochratoxin B apparently did not accumulate in any tissues, this indicated that ochratoxin B was completely hydrolysed in the body (perhaps by liver enzymes) and excreted as its metabolite, ochratoxin β. In common with the intestinal peptidases, liver enzymes metabolize ochratoxin B very much more rapidly than ochratoxin A (Doster & Sinnhuber, 1972); consequently very little of the latter toxin could have undergone degradation in the liver. This suggests, therefore, that where ochratoxin α was present in the urine, most of this metabolite was absorbed from the intestine before being excreted by the kidneys. By analogy with the behaviour of the parent toxins, it may be assumed that much less ochratoxin β was absorbed from the gut. Neither metabolite was detected in the serum but concentrations may have been below the limit of our analytical method (about 0·04 and 0·12 μg/ml serum for ochratoxins α and β, respectively).

These observations suggesting the preferential metabolism of ochratoxin B in the pig are consistent with the known relative affinities of carboxypeptidases for ochratoxins A and B (Doster & Sinnhuber, 1972); and the fact that ochratoxin B, the dechloroderivative of A, does not accumulate in tissues probably relates to the weaker dissociation of its phenolic hydroxyl group compared with that of ochratoxin A, the pK_a values being 8 and 7 respectively, (Chu, 1974; Chu *et al.* 1972) and hence to its weaker protein-binding properties.

The average daily intake of ochratoxin A by pigs in this experiment was some four times higher than the highest level in rations fed to pigs by Krogh *et al.* (1974) in a study of experimental nephropathy. Even so, their regression equations were used in the present experiment to predict from dietary concentrations of the toxin the accumulation of ochratoxin A in the liver and kidney and, as shown in Table 2, the calculated values were of the same order but from one half to one third of those actually determined at slaughter. Therefore, it was concluded that, in general, when concentrations of ochratoxin A in pig feed are known, the formulae of Krogh *et al.* (1974) could be used for the prediction of approximate levels of tissue contamination.

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MONOGRAPHS

Monographs on Fragrance Raw Materials*

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DIETHYL MALEATE

Synonym: Ethyl maleate.
Structure: $C_2H_5 \cdot OCO \cdot CH{:}CH \cdot OCO \cdot C_2H_5$.
Description and physical properties: A colourless liquid.
Occurrence: Has apparently not been reported to occur in nature.
Preparation: By direct esterification of maleic acid with ethyl alcohol (Arctander, 1969).
Uses: Use in fragrances in the USA amounts to less than 1000 lb/yr.
Concentration in final product (%):

	Soap	*Detergent*	*Creams, lotions*	*Perfume*
Usual	0·02	0·0033	0·01	0·1
Maximum	0·06	0·005	0·04	0·4

Status

Diethyl maleate is not included in the listings of the FDA, FEMA (1965) or the Council of Europe (1974), or in the *Food Chemicals Codex* (1972).

Biological data

Acute toxicity. The acute oral LD_{50} value in rats was reported as 3·2 g/kg (Fassett, 1963). The actue dermal LD_{50} value in rats was reported as >2·5 g/kg (Moreno, 1975) and as 5 ml/kg (Fassett, 1963).

Inhalation toxicity. No death occurred in rats exposed to saturated vapours of diethyl maleate for 8 hr (Fassett, 1963).

Irritation. Diethyl maleate applied full strength to intact or abraded rabbit skin for 24 hr under occlusion was moderately irritating (Moreno, 1975). The ester was reported to be slightly irritating to rabbit skin and eye (Fassett, 1963). Tested at 4% in petrolatum, it produced no irritation after a 48-hr closed-patch test on human subjects (Kligman, 1975).

Sensitization. A maximization test (Kligman, 1966; Kligman & Epstein, 1975) was carried out on 25 volunteers. The material was tested at a concentration of 4% in petrolatum and produced sensitization reactions in all 25 (Kligman, 1975). Diethyl maleate was reported to be a sensitizer in patch tests performed on four men working with unsaturated polyester resins (Malten & Zielhuis, 1964).

Metabolism. α,β-Unsaturated compounds, such as diethyl maleate, react enzymically with glutathione. The reaction has been demonstrated in fractions of rat liver (Boyland & Chasseaud, 1967) and avian liver (Wit & Snel, 1968). The enzyme differs from other known *S*-alkyl, *S*-aryl and *S*-epoxide transferase enzymes responsible for glutathione-conjugate formation (Boyland & Chasseaud, 1967). Diethyl maleate, administered parenterally to rats, reduced the hepatic glutathione content (Boyland & Chasseaud, 1970; Varga, Fischer & Szily, 1974). The latter workers also showed that diethyl maleate pretreatment of rats inhibited the glutathione conjugation of subsequently-administered bromsulphthalein.

Studying this ester's effect on the metabolism of parathion and methyl parathion, Mirer, Levine & Murphy (1975) showed that pretreatment of mice with diethyl maleate (1 mg/kg), 1 hr before challenge, depleted liver glutathione and potentiated parathion and methyl parathion toxicity. *In*

*The most recent of the previous sets of these monographs appeared in *Food and Cosmetics Toxicology* 1976, **14**, 307.

vivo, diethyl maleate potentiated the inhibition of brain cholinesterase by parathion and methyl parathion. Diethyl maleate pretreatment caused a twofold increase in the brain concentrations of parathion and methyl parathion and a large increase in the activation of methyl parathion to methyl paraoxon, and also decreased total degradation. Diethyl maleate inhibited the activation and degradation of parathion.

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OIL LAVANDIN ACETYLATED

Description and physical properties: The main constituent of oil lavandin acetylated is linalyl acetate (Arctander, 1960).
Occurrence: Has apparently not been reported to occur in nature.
Preparation: By acetylation of lavandin oil (Arctander, 1960).
Uses: In public use since the 1950s. Use of fragrances in the USA amounts to approximately 20,000 lb/yr.

Concentration in final product (%):

	Soap	*Detergent*	*Creams, lotions*	*Perfume*
Usual	0·03	0·003	0·015	0·03
Maximum	0·3	0·03	0·1	1·2

Analytical data: Gas chromatogram, RIFM nos 72-31, 72-176; infra-red curve, RIFM nos 72-31, 72-176.

Status

Oil lavandin acetylated is not included in the listings of the FDA, FEMA (1965) or the Council of Europe (1974), or in the *Food Chemicals Codex* (1972).

Biological data

Acute toxicity. Both the acute oral LD_{50} value in rats and the acute dermal LD_{50} value in rabbits exceeds 5 g/kg (Keating, 1972).

Irritation. Undiluted oil lavandin acetylated applied to the backs of hairless mice and swine was not irritating (Urbach & Forbes, 1972). Tested at 10% in petrolatum, it produced no irritation after a 48-hr closed-patch test on human subjects (Kligman, 1972).

Sensitization. A maximization test (Kligman, 1966; Kligman & Epstein, 1975) was carried out on 25 volunteers. The material was tested at a concentration of 10% in petrolatum and produced no sensitization reactions (Kligman, 1972).

Phototoxicity. No phototoxic effects were reported for undiluted oil lavandin acetylated on hairless mice and swine (Urbach & Forbes, 1972).

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LAVANDIN OIL

Description and physical properties: EOA Spec. no. 41. The main constituent of lavandin oil is linalool (*Fenaroli's Handbook of Flavor Ingredients*, 1971).
Occurrence: Found in the plant *Lavandula hybrida* Reverchon (Fam. Labiatae).
Preparation: By steam distillation of the flowering stalks of *Lavandula hybrida* Reverchon (Gildemeister & Hoffman, 1961).
Uses: In public use since the 1900s. Use in fragrances in the USA amounts to approximately 500.000 lb/yr.

Concentration in final product (%):

	Soap	*Detergent*	*Creams, lotions*	*Perfume*
Usual	0·03	0·003	0·015	0·3
Maximum	0·3	0·03	0·1	1·2

Analytical data: Gas chromatogram, RIFM no. 242981; infra-red curve, RIFM nos 242981, 73-24.

Status

Lavandin oil was given GRAS status by FEMA (1965) and is approved by the FDA for food use (GRAS).

Biological data

Acute toxicity. Both the acute oral LD_{50} value in rats and the acute dermal LD_{50} value in rabbits exceeded 5 g/kg (Moreno, 1973).

Irritation. Undiluted lavandin oil applied to the backs of hairless mice and swine was not irritating (Urbach & Forbes, 1973). Applied full strength to intact or abraded rabbit skin for 24 hr under occlusion, it was slightly irritating (Moreno, 1973). Tested at 5% in petrolatum, it produced no irritation after a 48-hr closed-patch test on human subjects (Kligman, 1971).

Sensitization. A maximization test (Kligman, 1966; Kligman & Epstein, 1975) was carried out on 26 volunteers. The material was tested at a concentration of 5% in petrolatum and produced no sensitization reactions (Kligman, 1971).

Phototoxicity. No phototoxic effects were reported for undiluted lavandin oil on hairless mice and swine (Urbach & Forbes, 1973).

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LAVENDER ABSOLUTE

Description and physical properties: A dark green liquid. The main constituent of lavender absolute is linalyl acetate (Guenther, 1949).
Occurrence: Found in the flowers of *Lavandula officinalis* chaix (Fam. Labiatae) (Guenther, 1949).
Preparation: From an alcoholic extract of the concrete which is extracted from the plant material using an organic solvent.
Uses: In public use since the 1920s. Use in fragrances in the USA amounts to approximately 5000 lb/yr.

Concentration in final product (%):

	Soap	*Detergent*	*Creams, lotions*	*Perfume*
Usual	0·01	0·001	0·005	0·2
Maximum	0·15	0·015	0·03	1·0

Analytical data: Gas chromatogram, RIFM no. NAK-3; infra-red curve, RIFM no. NAK-3.

Status

Lavender absolute was given GRAS status by FEMA (1965) and is approved by the FDA for food use (GRAS).

Biological data

Acute toxicity. The acute oral LD_{50} value in rats was reported as 4·25 g/kg (3·82–4·68 g/kg) (Moreno, 1975). The acute dermal LD_{50} value in guinea-pigs was reported at >5 g/kg (Moreno, 1975).

Irritation. Lavender absolute applied full strength to intact or abraded rabbit skin for 24 hr under occlusion was slightly irritating (Moreno, 1975). Tested at 10% in petrolatum, it produced no irritation after a 48-hr closed-patch test on human subjects (Kligman, 1975).

Sensitization. A maximization test (Kligman, 1966; Kligman & Epstein, 1975) was carried out on 25 volunteers. The material was tested at a concentration of 10% in petrolatum and produced no sensitization reactions (Kligman, 1975). Lavender absolute (referred to as lavender oil) has been listed as a sensitizer (Nakayama, Hanaoka & Ohshiro, 1974).

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LAVENDER OIL

Description and physical properties: *Food Chemicals Codex* (1972). The main constituent of lavender oil is linalyl acetate (Guenther, 1949).

Occurrence: Found in the plant *Lavandula officinalis* chaix (Fam. Labiatae) (Guenther, 1949).

Preparation: By steam distillation of the flowering stalks of *Lavandula officinalis* chaix (Guenther, 1949).

Uses: In public use since the 1920s. Use in fragrances in the USA amounts to approximately 100,000 lb/yr.

Concentration in final product (%):

	Soap	*Detergent*	*Creams, lotions*	*Perfume*
Usual	0·03	0·003	0·015	0·3
Maximum	0·3	0·03	0·1	1·2

Analytical data: Gas chromatogram, RIFM no. 73-25; infra-red curve, RIFM no. 73-25.

Status

Lavender oil was given GRAS status by FEMA (1965) and is approved by the FDA for food use (GRAS). The Council of Europe (1974) included lavender oil in the list of substances, spices and seasonings deemed admissible for use, with a possible limitation of the active principle in the final product. Both the *Food Chemicals Codex* (1972) and the *National Formulary* (1970) have monographs on lavender oil.

Biological data

Acute toxicity. Both the acute oral LD_{50} value in rats and the acute dermal LD_{50} value in rabbits exceeded 5 g/kg (Moreno, 1973).

Irritation. Undiluted lavender oil applied to the backs of hairless mice and swine was not irritating (Urbach & Forbes, 1973). Applied full strength to intact or abraded rabbit skin for 24 hr under occlusion, it was slightly irritating (Moreno, 1973). Tested at 16% in petrolatum, it produced no irritation after a 48-hr closed-patch test on human subjects (Kligman, 1971).

Sensitization. A maximization test (Kligman, 1966; Kligman & Epstein, 1975) was carried out on 25 volunteers. The material was tested at a concentration of 16% in petrolatum and produced no sensitization reactions (Kligman, 1971).

Phototoxicity. No phototoxic effects were reported for undiluted lavender oil on hairless mice and swine (Urbach & Forbes, 1973).

Percutaneous absorption. Lavender oil was not absorbed within 2 hr of application to the intact shaved abdominal skin of the mouse (Meyer & Meyer, 1959).

Additional published data

The chemistry of lavender oil has been reviewed extensively (de La Torre, 1974), and a study of lavender oils and their constitution has been published (Staicov, Chingova & Kalaidjiev, 1969).

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Urbach, F. & Forbes, P. D. (1973). Report to RIFM.

SPIKE LAVENDER OIL

Description and physical properties: EOA Spec. no. 4. The main constituents of spike lavender oil are linalool and cineole.
Occurrence: Found in the plant *Lavandula latifolia* Vill. (L. Spica D. C.) (Fam. Labiatae).
Preparation: By steam distillation of the sun-dried flowers.
Uses: In public use before the 1860s. Use in fragrances in the USA amounts to approximately 100,000 lb/yr.

Concentration in final product (%):

	Soap	*Detergent*	*Creams, lotions*	*Perfume*
Usual	0·03	0·03	0·01	0·2
Maximum	0·3	0·03	0·1	0·8

Analytical data: Gas chromatogram, RIFM no. 72-30; infra-red curve, RIFM no. 72-30.

Status

Spike lavender oil was given GRAS status by FEMA (1965) and is approved by the FDA for food use (GRAS). The Council of Europe (1974) included spike lavender oil in the list of substances, spices and seasonings deemed admissible for use, with a possible limitation of the active principle in the final product. The *Food Chemicals Codex* (1972) has a monograph on spike lavender oil.

Biological data

Acute toxicity. The acute oral LD_{50} value in rats was reported as 3·8 g/kg (3·3–4·3 g/kg) (Moreno, 1972a). The acute dermal LD_{50} value in rabbits was reported as >2 g/kg (Moreno, 1972b).

Irritation. Spike lavender oil applied full strength to intact or abraded rabbit skin for 24 hr under occlusion was moderately irritating (Moreno, 1972b). A patch test using spike lavender oil full strength for 24 hr produced no reactions in 15 human subjects (Katz, 1946). Tested at 8% in petrolatum, it produced no irritation after a 48-hr closed-patch test on human subjects (Kligman, 1972).

Sensitization. A maximization test (Kligman, 1966; Kligman & Epstein, 1975) was carried out on 25 volunteers. The material was tested at a concentration of 8% in petrolatum and produced no sensitization reactions (Kligman, 1972).

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LEMONGRASS OIL EAST INDIAN

Description and physical properties: EOA Spec. no. 7. The main constituent of lemongrass oil is citral (Guenther, 1950). The non-citral portion has been described by Aggar, Kamath & Rao (1968).
Occurrence: Found in the grasses of *Cymbopogon flexuosus* (Stapf) and *Andropogon nardus* var. flexuosus (Fam. Graminae).
Preparation: By steam distillation of the freshly cut and partially dried grasses (Gildemeister & Hoffman, 1956).
Uses: In public use since the 1920s. Use in fragrances in the USA amounts to approximately 50,000 lb/yr.

Concentration in final product (%):

	Soap	*Detergent*	*Creams, lotions*	*Perfume*
Usual	0·02	0·002	0·003	0·08
Maximum	0·25	0·025	0·02	0·7

Analytical data: Gas chromatogram, RIFM no. 71-48; infra-red curve, RIFM no. 71-48.

Status

Lemongrass oil was given GRAS status by FEMA (1965) and is approved by the FDA for food use (GRAS). The Council for Europe (1974) included lemongrass in the list of substances, spices and seasonings deemed admissible for use, with a possible limitation of the active principle in the final product. The *Food Chemicals Codex* (1972) has a monograph on lemongrass oil

Biological data

Acute toxicity. The acute oral LD_{50} value in rats was reported as 5·6 g/kg (5·1–6·1 g/kg) (Moreno, 1972a). The acute dermal LD_{50} value in rabbits exceeded 2 g/kg (Moreno, 1972b).

Irritation. Undiluted lemongrass oil E.I. applied to the backs of hairless mice and swine was mildly irritating (Urbach & Forbes, 1972). Applied full strength to intact or abraded rabbit skin for 24 hr under occlusion, it was moderately irritating (Moreno, 1972b). Tested at 4% in petrolatum, it produced no irritation after a 48-hr closed-patch test on human subjects (Kligman, 1972).

Sensitization. A maximization test (Kligman, 1966; Kligman & Epstein, 1975) was carried out on 25 volunteers. The material was tested at a concentration of 4% in petrolatum and produced no sensitization reactions (Kligman, 1972).

Phototoxicity. No phototoxic effects were reported for undiluted lemongrass oil E.I. on hairless mice and swine (Urbach & Forbes, 1972).

References

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Urbach. F. & Forbes, P. D. (1972). Report to RIFM, 26 May.

LEMONGRASS OIL WEST INDIAN

Description and physical properties: EOA Spec. no. 7. The main constituent of lemongrass oil W.I. is citral (Guenther, 1950).
Occurrence: Found in the grasses of *Cymbopogon citratus* (Stapf) and *Andropogon nardus* var. ceriferus (Hack).
Preparation: By steam distillation of the freshly cut and partially dried grasses (Gildemeister & Hoffman, 1956).
Uses: In public use since the 1920s. Use in fragrances in the USA amounts to approximately 250,000 lb/yr.

Concentration in final product (%):

	Soap	*Detergent*	*Creams, lotions*	*Perfume*
Usual	0·02	0·002	0·003	0·08
Maximum	0·25	0·025	0·02	0·7

Analytical data: Gas chromatogram, RIFM no. 71-4; infra-red curve, RIFM no. 71–4.

Status

Lemongrass oil was given GRAS status by FEMA (1965) and is approved by the FDA for food use (GRAS). The Council of Europe (1974) included lemongrass in the list of substances, spices and seasonings deemed admissible for use, with a possible limitation of the active principle in the final product. The *Food Chemicals Codex* (1972) has a monograph on lemongrass oil.

Biological data

Acute toxicity. Both the acute oral LD_{50} value in rats and the acute dermal LD_{50} value in rabbits exceeded 5 g/kg (Hart, 1971).

Irritation. Lemongrass oil W.I. applied undiluted to the backs of hairless mice and swine was mildly irritating (Urbach & Forbes, 1972). Applied full strength to intact or abraded rabbit skin for 24 hr under occlusion, it was moderately irritating (Hart, 1971). Tested at 4% in petrolatum, the material (RIFM no 71-4-4) produced no irritation after a 48-hr closed-patch test on human subjects (Kligman, 1971). Lemongrass oil W.I. (RIFM no. DL-5-01) tested at 5% in petrolatum similarly produced no irritation after a 48-hr closed-patch test on human subjects (Kligman, 1972).

Sensitization. A maximization test (Kligman, 1966; Kligman & Epstein, 1975) was carried out on 25 volunteers. The material (RIFM no. 71-4-4) was tested at a concentration of 4% in petrolatum and produced no sensitization reactions (Kligman, 1971). In a further maximization test on 25 volunteers, the material (RIFM no. DL-5-01) was tested at a concentration of 5% in petrolatum and produced no sensitization reactions (Kligman, 1972).

Phototoxicity. No phototoxic effects were reported for undiluted lemongrass oil W.I. on hairless mice and swine (Urbach & Forbes, 1972).

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LINALYL ANTHRANILATE

Synonym: Linalyl *o*-aminobenzoate.
Structure: $CH_3 \cdot C(CH_3):CH \cdot [CH_2]_2 \cdot (CH_3)C(CH:CH_2) \cdot OCO \cdot C_6H_4 \cdot NH_2$.
Description and physical properties: A pale straw-coloured oily liquid.
Occurrence: Has apparently not been reported to occur in nature.
Preparation: From linalyl formate plus methyl anthranilate with sodium linalool or from linalool plus isatoic anhydride with a trace of sodium hydroxide as starter catalyst, or by any other suitable means.
Uses: In public use since the 1930s. Use in fragrances in the USA amounts to less than 1000 lb/yr.
Concentration in final product (%):

	Soap	*Detergent*	*Creams, lotions*	*Perfume*
Usual	0·01	0·01	0·005	0·12
Maximum	0·2	0·02	0·2	0·8

Analytical data: Gas chromatograms, RIFM no. 72-178; infra-red curve, RIFM no. 72-178.

Status

Linalyl anthranilate was given GRAS status by FEMA (1965) and is approved by the FDA for food use (21 CFR 121.1164). The Council of Europe (1974) included linalyl anthranilate at a level of 8 ppm in the list of artificial flavouring substances that may be added to foodstuffs without hazard to public health.

Biological data

Acute toxicity. The acute oral LD_{50} value in rats was reported as 4·25 g/kg (4·0–4·5 g/kg) (Russell, 1973). The acute dermal LD_{50} value in rabbits was reported as >5 g/kg (Russell, 1973).

Irritation. Linalyl anthranilate applied full strength to intact or abraded rabbit skin for 24 hr under occlusion was not irritating (Russell, 1973). Tested at 8% in petrolatum, it produced no irritation after a 48-hr closed-patch test on human subjects (Kligman, 1973).

Sensitization. A maximization test (Kligman, 1966; Kligman & Epstein, 1975) was carried out on 25 volunteers. The material was tested at a concentration of 8% in petrolatum and produced no sensitization reactions (Kligman, 1973).

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Kligman, A. M. & Epstein, W. (1975). Updating the maximization test for identifying contact allergens. *Contact Dermatitis* **1**, 231.
Russell, T. J. (1973). Report to RIFM, 6 March.

LINALYL BENZOATE

Synonym: 3,7-Dimethyl-1,6-octadien-3-yl benzoate.
Structure: $CH_3 \cdot C(CH_3):CH \cdot [CH_2]_2 \cdot (CH_3)C(CH:CH_2) \cdot OCO \cdot C_6H_5$.
Description and physical properties: *Givaudan Index* (1961).
Occurrence: Reported to be found in the essential oils of ylang-ylang and tuberose (*Fenaroli's Handbook of Flavor Ingredients*, 1971).
Preparation: By esterification of dehydrolinalool with benzoic acid followed by hydrogenation of the dehydro ester, or by any other suitable means.
Uses: In public use since the 1920s. Use in fragrances in the USA amounts to less than 3000 lb/yr.
Concentration in final product (%):

	Soap	*Detergent*	*Creams, lotions*	*Perfume*
Usual	0·03	0·003	0·01	0·12
Maximum	0·2	0·02	0·06	0·8

Status

Linalyl benzoate was given GRAS status by FEMA (1965) and is approved by the FDA for food use (21 CFR 121.1164). The Council of Europe (1974) included linalyl benzoate at a level of 1 ppm in the list of artificial flavouring substances that may be added to foodstuffs without hazard to public health. The *Food Chemicals Codex* (1972) has a monograph on linalyl benzoate.

Biological data

Acute toxicity. Both the acute oral LD_{50} value in rats and the acute dermal LD_{50} value in rabbits exceeded 5 g/kg (Moreno, 1973).

Irritation. Linalyl benzoate applied full strength to intact or abraded rabbit skin for 24 hr under occlusion was mildly irritating (Moreni, 1973). Tested at 8% in petrolatum, it produced no irritation after a 48-hr closed-patch test on human subjects (Kligman, 1973).

Sensitization. A maximization test (Kligman, 1966; Kligman & Epstein, 1975) was carried out on 25 volunteers. The material was tested at a concentration of 8% in petrolatum and produced no sensitization reactions (Kligman, 1973).

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Moreno, O. M. (1973). Report to RIFM, 18 May.

LINALYL CINNAMATE

Structure: $CH_3 \cdot C(CH_3):CH \cdot [CH_2]_2 \cdot (CH_3)C(CH:CH_2) \cdot OCO \cdot CH:CH \cdot C_6H_5$.
Description and physical properties: An almost colourless oily or slightly viscous liquid.
Occurrence: Has apparently not been reported to occur in nature.
Preparation: From linalyl formate plus methyl cinnamate plus linalool sodium, or from dehydrolinalool and cinnamic acid via the dehydro ester, which is hydrogenated to the subject ester, or by any other suitable means.
Uses: In public use since the 1920s. Use in fragrances in the USA amounts to less than 3000 lb/yr.
Concentration in final product (%):

	Soap	*Detergent*	*Creams, lotions*	*Perfume*
Usual	0·01	0·001	0·005	0·12
Maximum	0·1	0·01	0·03	0·8

Status

Linalyl cinnamate was given GRAS status by FEMA (1965) and is approved by the FDA for food use (21 CFR 121.1164). The Council of Europe (1974) listed linalyl cinnamate giving an ADI of 1·25 mg/kg.

Biological data

Acute toxicity. The acute oral LD_{50} value in rats was reported as 9·96 g/kg (Jenner, Hagan, Taylor, Cook & Fitzhugh, 1964). The acute dermal LD_{50} value in rabbits exceeded 5 g/kg (Moreno, 1973).

Subacute toxicity. In feeding studies, 1000, 2500 and 10,000 ppm linalyl cinnamate fed to rats in the diet for 17 wk produced no effects (Hagan, Hansen, Fitzhugh, Jenner, Jones, Taylor, Long Nelson & Brouwer, 1967).

Irritation. Linalyl cinnamate applied full strength to intact or abraded rabbit skin for 24 hr under occlusion was mildly irritating (Moreno, 1973). Tested at 8% in petrolatum, it produced no irritation after a 48-hr closed-patch test on human subjects (Kligman, 1973).

Sensitization. A maximization test (Kligman, 1966; Kligman & Epstein, 1975) was carried out on 25 volunteers. The material was tested at a concentration of 8% in petrolatum and produced no sensitization reactions (Kligman, 1973).

References

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Hagan, E. C., Hansen, W. H., Fitzhugh, O. G., Jenner, P. M., Jones, W. I., Taylor, J. M., Long, E. L., Nelson, A. A. & Brouwer, J. B. (1967). Food flavourings and compounds of related structure. II. Subacute and chronic toxicity. *Fd Cosmet. Toxicol.* **5**, 141.
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Kligman, A. M. (1973). Report to RIFM, 9 October.
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Moreno, O. M. (1973). Report to RIFM, 14 May.

LINALYL PHENYLACETATE

Synonym: Linalyl α-toluate.
Structure: $CH_3 \cdot C(CH_3):CH \cdot [CH_2]_2 \cdot (CH_3)C(CH:CH_2) \cdot OCO \cdot CH_2 \cdot C_6H_5$.
Description and physical properties: A colourless or pale straw-coloured viscous liquid.
Occurrence: Has apparently not been reported to occur in nature.
Preparation: From dehydrolinalool and methyl phenylacetate in the presence of sodium methylate catalyst, followed by hydrogenation of the ester, or by any other suitable means.
Uses: In public use since the 1930s. Use in fragrances in the USA amounts to less than 1000 lb/yr.
Concentration in final product (%):

	Soap	*Detergent*	*Creams, lotions*	*Perfume*
Usual	0·01	0·001	0·005	0·12
Maximum	0·1	0·01	0·01	0·4

Status

The Council of Europe (1974) has included linalyl phenylacetate at a level of 0·5 ppm in the list of artificial flavouring susbstances that may be added to foodstuffs without hazard to public health.

Biological data

Acute toxicity. Both the acute oral LD_{50} value in rats and the acute dermal LD_{50} value in rabbits exceeded 5 g/kg (Moreno, 1974).

Irritation. Linalyl phenylacetate applied full strength to intact or abraded rabbit skin for 24 hr under occlusion was not irritating (Moreno, 1974). Tested at 4% in petrolatum, it produced no irritation after a 48-hr closed-patch test on human subjects (Kligman, 1974).

Sensitization. A maximization test (Kligman, 1966; Kligman & Epstein, 1975) was carried out on 25 volunteers. The material was tested at a concentration of 4% in petrolatum and produced no sensitization reactions (Kligman, 1974).

Metabolism: Open-chain olefinic terpene esters are presumably hydrolysed to the alcohol and the acid (Fassett, 1963). Open-chain terpenes are metabolized in the rabbit by ω-oxidation and by reduction of an α,β-double bond (Williams, 1959).

References

Council of Europe (1974). Natural Flavouring Substances, Their Sources, and Added Artificial Flavouring Substances. Partial Agreement in the Social and Public Health Field. List 1, no. 655, p. 264. Strasbourg.

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Kligman, A. M. & Epstein, W. (1975). Updating the maximization test for identifying contact allergens. *Contact Dermatitis* **1**, 231,

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MARJORAM OIL, SPANISH

Description and physical properties: EOA Spec. no. 143. The main constituent of marjoram oil, Spanish is cineole (Guenther, 1949).
Occurrence: Found in the shrub *Thymus mastichina* L. (Fam. Labiatae).
Preparation: By steam-distillation of the flowering plant material *Thymus mastichina* L.
Uses: In public use before the 1900s. Use in fragrances in the USA amounts to less than 2000 lb/yr.
Concentration in final product (%):

	Soap	*Detergent*	*Creams, lotions*	*Perfume*
Usual	0·02	0·003	0·005	0·12
Maximum	0·15	0·015	0·03	0·6

Analytical data: Gas chromatogram, RIFM no. 72-185; infra-red curve, RIFM, no. 72-185.

Status

The *Food Chemicals Codex* (1972) has a monograph on marjoram oil, Spanish.

Biological data

Acute toxicity. Both the acute oral LD_{50} value in rats and the acute dermal LD_{50} value in rabbits exceeded 5 g/kg (Moreno, 1973).

Irritation. Marjoram oil, Spanish applied full strength to intact or abraded rabbit skin for 24 hr under occlusion was mildly irritating (Moreno, 1973). Applied undiluted to the backs of hairless mice and swine, it was not irritating (Urbach & Forbes, 1973), and tested at 6% in petrolatum, it produced no irritation after a 48-hr closed-patch test on human subjects (Epstein, 1973).

Sensitization. A maximization test (Kligman, 1966; Kligman & Epstein, 1975) was carried out on 23 human volunteers. The material was tested at a concentration of 6% in petrolatum and produced no sensitization reactions (Epstein, 1973).

Phototoxicity. No phototoxic effects were reported for undiluted marjoram oil, Spanish on hairless mice and swine (Urbach & Forbes, 1973).

References

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Food Chemicals Codex (1972). 2nd Ed. Prepared by the Committee on Specifications, Food Chemicals Codex, of the Committee on Food Protection. p. 499. National Academy of Sciences–National Research Council, Washington, D.C.
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Kligman, A. M. & Epstein, W. (1975). Updating the maximization test for identifying contact allergens. *Contact Dermatitis* **1**, 231.
Moreno, O. M. (1973). Report to RIFM, 14 May.
Urbach, F. & Forbes, P. D. (1973). Report to RIFM, 7 May.

MARJORAM OIL SWEET

Description and physical properties: A pale yellow mobile liquid. The main constituents of marjoram oil sweet include terpinene and terpineol (Guenther, 1949).
Occurrence: Found in the plant *Marjorana hortensis* Moench (*Origanum marjorana* L.; Fam. Labiatae) (Guenther, 1949).
Preparation: By steam-distillation of the leaves of the plant *Origanum marjorana* L. (Guenther, 1949).
Uses: In public use before the 1880s. Use in fragrances in the USA amounts to less than 1000 lb/yr.
Concentration in final product (%):

	Soap	*Detergent*	*Creams, lotions*	*Perfume*
Usual	0·02	0·003	0·005	0·12
Maximum	0·15	0·015	0·03	0·6

Analytical data: Gas chromatogram, RIFM no. 73-184; infra-red curve, RIFM no. 73-184.

Status

Marjoram oil sweet was given GRAS status by FEMA (1965) and is approved by the FDA for food use (GRAS). The Council of Europe (1974) included marjoram oil sweet in the list of substances, spices and seasonings deemed admissible for use, with a possible limitation of the active principle in the final product.

Biological data

Acute toxicity. The acute oral LD_{50} value in rats was reported as 2·24 g/kg (Wohl, 1974). The acute dermal LD_{50} value in rabbits exceeded 5 g/kg (Wohl, 1974).

Irritation. Marjoram oil sweet applied full strength to intact or abraded rabbit skin for 24 hr under occlusion was not irritating (Wohl, 1974). Tested at 6% in petrolatum, it produced no irritation after a 48-hr closed-patch test on human subjects (Epstein, 1973).

Sensitization. A maximization test (Kligman, 1966; Kligman & Epstein, 1975) was carried out on 23 human volunteers. The material was tested at a concentration of 6% in petrolatum and produced no sensitization reactions (Epstein, 1973).

References

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Epstein, W. L. (1973). Report to RIFM, 1 October.

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Guenther, E. (1949). *The Essential Oils.* Vol. III. p. 519. D. Van Nostrand, Inc., Princeton, New Jersey.

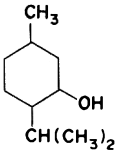
Kligman, A. M. (1966). The identification of contact allergens by human assay. III. The maximization test. A procedure for screening and rating contact sensitizers. *J. invest. Derm.* **47**, 393.

Kligman, A. M. & Epstein, W. (1975). Updating the maximization test for identifying contact allergens. *Contact Dermatitis* **1**, 231.

Wohl, A. J. (1974). Report to RIFM, 2 April.

l-MENTHOL

Synonyms: 3-*p*-Menthanol; *p*-menthan-3-ol; 4-isopropyl-1-methylcyclohexan-3-ol.
Structure:



Description and physical properties: *Givaudan Index* (1961).
Occurrence: Found in high concentrations in oils of peppermint (*Mentha piperita*) and Japanese mint oil (*Mentha arvensis*) and in lower concentrations in Réunion geranium oil, and also in a large number of essential oils (*Fenaroli's Handbook of Flavor Ingredients*, 1971; Guenther, 1949).
Preparation: By isolation from *Mentha arvensis* oils or synthetically from turpentine and from thymol.
Uses: In public use before the 1900s. Use in fragrances in the USA amounts to less than 50,000 lb/yr.
Concentration in final product (%):

	Soap	*Detergent*	*Creams, lotions*	*Perfume*
Usual	0·02	0·002	0·01	0·04
Maximum	0·2	0·02	0·05	0·3

Status

Menthol was given GRAS status by FEMA (1965) and is approved by the FDA for food use (21 CFR 121.1164). The Council of Europe (1974) listed menthol, giving an ADI of 2 mg/kg. The *Food Chemicals Codex* (1972) and the *United States Pharmacopeia* (1965) have monographs on menthol. The Joint FAO/WHO Expert Committee on Food Additives (1968) has published a monograph and specifications for menthol giving an unconditional ADI of 0–0·2 mg/kg.

Biological data

Acute toxicity. The acute oral LD_{50} values were reported as 3300 mg/kg in the rat (Herken, 1961) and as 800–1000 mg/kg in the cat (Flury, 1920). The sc LD_{50}s in mice and rats were reported at 5000–6000 and 1000–2500 mg/kg, respectively (Flury, 1920). The lethal sc dose in rats was reported as 2000 mg/kg (*Merck Index*, 1968). The lethal ip doses in mice, rats and guinea-pigs were reported as 2000, 1500 and 4000 mg/kg, respectively (Macht, 1939) while the ip LD_{50} in cats was reported as 800–1000 mg/kg (Flury, 1920) and that in rabbits as approximately 2000 mg/kg (Herken, 1961). The lethal iv dose in cats was reported at 34 mg/kg (Macht, 1939), and the acute dermal LD_{50} value in rabbits as >5 g/kg (Wohl, 1974).

Short-term toxicity. In a feeding study, groups of 40 male and 40 female rats received 0, 100 or 200 mg/kg body weight of either *l*- or *dl*-menthol in their diets for 5·5 wk. There were no adverse efffects on weight gain or excretion of glucuronide, water and electrolytes, nor was there any interference with CNS reactions to cardrazol or electric shock or with iv hexobarbitone sleeping-time compared with controls (Herken, 1961).

Menthol has a psycholeptic effect in the mouse. However, the psychotropic activity of this compound, particularly when administered orally, is weak. When administered ip, the effective dose is of the order of 100–600 mg/kg, according to the test used and the substance studied. The psychotropic action of this substance is always transient and it is unlikely that it would have any effects of this kind in man at the concentrations that occur in food or drinks (Le Bourhis & Soenen, 1973).

Menthol was studied to establish whether it could affect the metabolism of other drugs in rats and was found to be inactive with respect to pentobarbitone sleeping time (Jori, Bianchetti & Prestini, 1969).

Chronic urticaria with basophil leucopaenia on challenge has been reported after contact with menthol in toothpaste, mentholated cigarettes and peppermint sweets (McGowan, 1966). Bradycardia, ataxia, confusion and mental irritability were correlated with the inhalation of volatile menthol (from mentholated cigarettes) in a 58-yr-old woman (Luke, 1962). Two cases of idiopathic auricular fibrillation from excessive peppermint eating have been reported (Thomas, 1962), and a case of generalized urticaria in a young woman resulting from a predilection for peppermint candy, mint-flavoured toothpaste and mentholated cigarettes has been reported (Papa & Shelley, 1964). Strict avoidance of peppermint and all other sources of menthol led to the prompt disappearance of effects, after which the patient reacted positively to challenge tests by menthol, both dermal and oral.

Irritation. *l*-Menthol applied full strength to intact or abraded rabbit skin for 24 hr under occlusion was not irritating (Wohl, 1974). Tested at 8% in petrolatum, it produced no irritation after a 48-hr

closed-patch test in human subjects (Epstein, 1974), although it has been reported that menthol may act as a mild irritant in man (*Merck Index*, 1968).

Sensitization. A maximization test (Kligman, 1966; Kligman & Epstein, 1975) was carried out on 24 volunteers. The material was tested at a concentration of 8% in petrolatum and produced no sensitization reactions (Epstein, 1974).

Metabolism. *l*-Menthol conjugates readily in the rabbit forming *l*-menthyl-*β*-*d*-glucuronide. About half of the *l*-menthol fed to rabbits is excreted combined with glucuronic acid (Williams, 1938); the fate of the other half is not known, but it is possible that ring fission occurs with considerable degradation of the menthol molecule. In dogs, much oxidation of menthol takes place and only about 5% of the dose can be recovered in the urine as the glucuronide. According to Quick (1924) the percentage of a dose of *l*-menthol that is excreted combined with glucuronic acid in the rabbit depends on the magnitude of the dose; the larger the dose, the less is the conjugation (Williams, 1959).

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Flury, F. (1920). *Abderhaldens Handbuch der biologischen Arbeitsmethoden* **39,** 1365. Cited from Joint FAO/WHO Expert Committee on Food Additives (1968).

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Merck Index (1968). *An Encyclopedia of Chemicals and Drugs.* 8th Ed., p. 653. Merck & Co., Inc., Rahway, New Jersey.

Papa, C. M. & Shelley, W. B. (1964). Menthol hypersensitivity. Diagnostic basophil response in a patient with chronic urticaria, flushing and headaches. *J. Am. med. Ass.* **189,** 546.

Quick, A. J. (1924). *J. biol. Chem.* **61,** 681. Cited from Joint FAO/WHO Expert Committee on Food Additives (1968).

Thomas, J. G. (1962). Peppermint fibrillation. *Lancet* **i,** 222.

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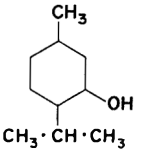
Williams, R. T. (1938). Studies in detoxication. II. (a) The conjugation of isomeric 3-menthanols with glucuronic acid and the asymmetric conjugation of *dl*-menthol and *dl-iso*menthol in the rabbit. (b) *d-iso*Menthylglucuronide, a new conjugated glucuronic acid. *Biochem. J.* **32,** 1849.

Williams, R. T. (1959). *Detoxication Mechanisms. The Metabolism and Detoxication of Drugs, Toxic Substances and Other Organic Compounds.* 2nd Ed., p. 528. Chapman & Hall Ltd., London.

Wohl, A. J. (1974). Report to RIFM, 15 May.

MENTHOL RACEMIC

Synonyms: *dl*-Menthol; 3-*p*-menthanol; 4-isopropyl-1-methylcyclohexan-3-ol.
Structure:



Description and physical properties: *Givaudan Index* (1961).
Occurrence: Has apparently not been reported to occur in nature.
Preparation: By hydrogenation of thymol followed by separation from its other isomers.
Uses: In public use since the 1900s. Use in fragrances in the USA amounts to approximately 5000 lb/yr.

Concentration in final product (%):

	Soap	*Detergent*	*Creams, lotions*	*Perfume*
Usual	0·03	0·003	0·01	0·04
Maximum	0·2	0·02	0·05	0·3

Analytical data: Gas chromatogram, RIFM no. 72-187; infra-red curve, RIFM no. 72-187.

Status

Menthol was given GRAS status by FEMA (1965) and is approved by the FDA for food use (21 CFR 121.1164). The Council of Europe (1974) listed menthol, giving an ADI of 2 mg/kg. The *Food Chemicals Codex* (1972) and the *United States Pharmacopeia* (1965) have monographs on menthol. The Joint FAO/WHO Expert Committee on Food Additives (1967) has published a monograph and specifications for menthol, giving an unconditional ADI of 0–0·2 mg/kg.

Biological data

Acute toxicity. The acute oral LD_{50} in rats has been reported as 3180 mg/kg by Jenner, Hagan, Taylor, Cook & Fitzhugh (1964) and as 2900 mg/kg by Herken (1961). The acute oral LD_{50} in cats was reported to be 1500–1600 mg/kg (Flury & Seel, 1926). The sc LD_{50} in the mouse was reported as 1400–1600 mg/kg (Flury & Seel, 1926) and the ip LD_{50} as 750 mg/kg in the rat (Herken, 1961) and 1500–1600 mg/kg in the cat (Flury & Seel, 1926). In rabbits, the ip LD_{50} was reported to be approximately 2000 mg/kg (Herken, 1961), while the acute dermal LD_{50} exceeded 5000 mg/kg (Levenstein, 1973).

Short-term toxicity. In a feeding study, groups of 40 male and 40 female rats recieved 0, 100 or 200 mg/kg body weight of either *l*- or *dl*-menthol in their diets for 5·5 wk. There were no adverse effects on weight gain or excretion of glucuronide, water and electrolytes, nor was there any interference with CNS reactions to cardrazol or electric shock or with iv hexobarbitone sleeping-time compared with controls (Herken, 1961).

Irritation. Menthol racemic applied full strength to intact or abraded rabbit skin for 24 hr under occlusion was mildly irritating (Levenstein, 1973). Tested at 8% in petrolatum, it produced no irritation after a 48-hr closed-patch test in human subjects (Kligman, 1973).

Sensitization. A maximization test (Kligman, 1966; Kligman & Epstein, 1975) was carried out on 25 volunteers. The material was tested at a concentration of 8% in petrolatum and produced no sensitization reactions (Kligman, 1973).

Metabolism. Rabbits are said to eliminate 59% of *dl*-menthol as glucuronide (Williams, 1938).

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Herken, H. (1961). Report to Schering AG. Cited from Joint FAO/WHO Expert Committee on Food Additives (1968).

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Joint FAO/WHO Expert Committee on Food Additives (1968). Toxicological Evaluation of Some Flavouring Substances and Non-nutritive Sweetening Agents. WHO/Food Add./68.33; F.A.O. Nutr. Mtg Rep. Ser. no. 44A, Rome, p. 58.

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Levenstein, I. (1973). Report to RIFM, 16 February.

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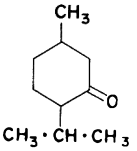
Williams, R. T. (1938). Studies in detoxication. II. (a) The conjugation of isomeric 3-menthanols with glucuronic acid and the asymmetric conjugation of *dl*-menthol and *dl-iso*menthol in the rabbit. (b) *d-iso*Menthyl-glucuronide, a new conjugated glucuronic acid. *Biochem. J.* **32,** 1849.

MENTHONE RACEMIC

Synonyms: 4-Isopropyl-1-methylcyclohexan-3-one; *p*-menthan-3-one.
Structure:



Description and physical properties: *Givaudan Index* (1961).
Occurrence: Several stereoisomers are reported to be found in nature. *l*-Menthone is a constituent of the essential oils of Russian and American peppermint, geranium, *Andropogon fragrans*, *Mentha timija*, *Mentha arvensis* and others. *d*-Menthone is present in the essential oils of *Barosma pulchellum*, *Nepeta japonica* Maxim. and others. *d*-Isomenthone has been reported to be isolated from *Micromeria abissinica* Benth., *Pelargonium tomentosum* Jacquin. and others. *l*-Isomenthone has been identified in Reunion geranium, *Pelargonium capitatum* and others (*Fenaroli's Handbook of Flavor Ingredients*, 1971).
Preparation: By oxidation of menthol or by partial hydrogenation of thymol.
Uses: In public use since the 1920s. Use of fragrances in the USA amounts to approximately 1000 lb/yr.

Concentration in final product (%):

	Soap	*Detergent*	*Creams, lotions*	*Perfume*
Usual	0·02	0·002	0·01	0·1
Maximum	0·2	0·02	0·05	0·8

Analytical data: Gas chromatogram, RIFM no. 72-188; intra-red curve, RIFM no. 72-188.

Status

Menthone was given GRAS status by FEMA (1965) and is approved by the FDA for food use (21 CFR 121.1164). The Council of Europe (1974) included menthone in the list of artificial flavouring substances that may be added temporarily to foodstuffs without hazard to public health.

Biological data

Acute toxicity. The acute oral LD_{50} in rats was reported as 2·18 ml/kg (1·82–2·62 ml/kg) (Levenstein, 1973a). The acute dermal LD_{50} in rabbits exceeded 5 g/kg (Levenstein, 1973b).

Irritation. Menthone racemic applied full strength to intact or abraded rabbit skin for 24 hr under occlusion was mildly irritating (Levenstein, 1973b). Tested at 8% in petrolatum, it produced no irritation after a 48-hr closed-patch test in human subjects (Kligman, 1973).

Sensitization. A maximization test (Kligman, 1966; Kligman & Epstein, 1975) was carried out on 25 volunteers. The material was tested at a concentration of 8% in petrolatum and produced no sensitization reactions (Kligman, 1973).

Metabolism. Hämäläinen (1912) claimed that menthone was probably oxidized in the rabbit to ketomenthone, since the glucuronide produced was converted by warm dilute H_2SO_4 to a substance which was probably $\Delta^{4(8)}$-*p*-menthen-3-one (pulegone). Williams (1940), however, showed that it underwent reduction in the rabbit with the production of *d*-neomenthol. The presence of *l*-menthol, the other possible reduction product, was not detected and it appeared that the biological reduction was asymmetric (Williams, 1959).

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l-MENTHYL ACETATE

Synonym: *l-p*-Menth-3-yl acetate.
Structure: $(CH_3)_2 \cdot CH \cdot C_6H_9(CH_3) \cdot OCO \cdot CH_3$.
Description and physical properties: *Merck Index* (1968).
Occurrence: Found in peppermint oil (Guenther, 1949).
Preparation: By direct esterification of *l*-menthol with acetic acid under azeotropic conditions (Arctander, 1969).
Uses: In public use since the 1930s. Use in fragrances in the USA amounts to approximately 3000 lb/yr.

Concentration in final product (%):

	Soap	*Detergent*	*Creams, lotions*	*Perfume*
Usual	0·02	0·002	0·01	0·2
Maximum	0·2	0·02	0·05	0·8

Analytical data: Gas chromatogram, RIFM no. 72-189; infra-red curve, RIFM no. 72-189.

Status

Menthyl acetate was given GRAS status by FEMA (1965) and is approved by the FDA for food use (21 CFR. 121.1164). The Council of Europe (1974) listed menthyl acetate giving an ADI of 2 mg/kg (therapeutic doses).

Biological data

Acute toxicity. Both the acute oral LD_{50} in rats and the acute dermal LD_{50} in rabbits exceeded 5 g/kg (Shelanski, 1972).

Irritation. *l*-Menthyl acetate applied full strength to intact or abraded rabbit skin for 24 hr under occlusion was mildly irritating (Shelanski, 1972). Tested at 8% in petrolatum, it produced no irritation after a 48-hr closed-patch test on human subjects (Kligman, 1972).

Sensitization. A maximization test (Kligman, 1966; Kligman & Epstein, 1975) was carried out on 25 volunteers. The material was tested at a concentration of 8% in petrolatum and produced no sensitization reactions (Kligman, 1972).

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MENTHYL ACETATE RACEMIC

Synonym: *dl*-Menthyl acetate.
Structure: $(CH_3)_2 \cdot CH \cdot C_6H_9(CH_3) \cdot OCO \cdot CH_3$.
Description and physical properties: *Givaudan Index* (1961).
Occurrence: Reported to be found in peppermint oil (Guenther, 1949).
Preparation: By direct esterification of racemic menthol with acetic acid under azeotropic conditions (Arctander, 1969).
Uses: In public use since the 1930s. Use in fragrances in the USA amounts to approximately 3000 lb/yr.

Concentration in final product (%):

	Soap	*Detergent*	*Creams, lotions*	*Perfume*
Usual	0·02	0·002	0·01	0·2
Maximum	0·2	0·02	0·05	0·8

Analytical data: Gas chromatogram, RIFM no. 72-190; infra-red curve, RIFM no. 72-190.

Status

Menthyl acetate was given GRAS status by FEMA (1965) and is approved by the FDA for food use (21 CFR 121.1164). The Council of Europe (1974) listed menthyl acetate, giving an ADI of 2 mg/kg (therapeutic doses).

Biological data

Acute toxicity. The acute oral LD_{50} in rats was reported as 7·62 ml/kg (5·95–9·75 ml/kg) (Levenstein, 1973a). The acute dermal LD_{50} in rabbits was reported as >5 g/kg (Levenstein, 1973b).

Irritation. Racemic menthyl acetate applied full strength to intact or abraded rabbit skin for 24 hr under occlusion was mildly irritating (Levenstein, 1973b). Tested at 8% in petrolatum, it produced no irritation after a 48-hr closed-patch test on human subjects (Kligman, 1973).

Sensitization. A maximization test (Kligman, 1966; Kligman & Epstein, 1975) was carried out on 25 volunteers. The material was tested at a concentration of 8% in petrolatum and produced no sensitization reactions (Kligman, 1973).

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Levenstein, I. (1973a). Report to RIFM, 10 January.

Levenstein, I. (1973b). Report to RIFM, 16 February.

METHYL ANISATE

Synonym: Methyl-*p*-methoxybenzoate.
Structure: $CH_3 \cdot O \cdot C_6H_4 \cdot OCO \cdot CH_3$.
Description and physical properties: *Givaudan Index* (1961).
Occurrence: Reported to be found in the mushroom, *Trametes graveolens* (*Fenaroli's Handbook of Flavor Ingredients*, 1971).
Preparation: By direct esterification of anisic acid with methyl alcohol, e.g. under azeotropic conditions (Arctander, 1969).
Uses: In public use before the 1920s. Use in fragrances in the USA amounts to less than 1000 lb/yr. Concentration in final product (%):

	Soap	*Detergent*	*Creams, lotions*	*Perfume*
Usual	0·01	0·001	0·005	0·12
Maximum	0·1	0·01	0·03	0·4

Status

Methyl anisate was given GRAS status by FEMA (1965) and is approved by the FDA for food use (21 CFR 121.1164). The Council of Europe (1974) included methyl anisate at a level of 8 ppm in the list of artificial flavouring substances that may be added to foodstuffs without hazard to public health.

Biological data

Acute toxicity. Both the acute oral LD_{50} in rats and the acute dermal LD_{50} in rabbits exceeded 5 g/kg (Levenstein, 1975).

Irritation. Methyl anisate applied full strength to intact or abraded rabbit skin for 24 hr under occlusion was mildly irritating (Levenstein, 1975). Tested at 4% in petrolatum, it produced no irritation after a 48-hr closed-patch test on human subjects (Kligman, 1975).

Sensitization. A maximization test (Kligman, 1966; Kligman & Epstein, 1975) was carried out on 25 volunteers. The material was tested at a concentration of 4% in petrolatum and produced no sensitization reactions (Kligman, 1975).

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Review Section

RECENT STUDIES OF LYSINOALANINE IN ALKALI-TREATED PROTEINS

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Summary—Lysinoalanine (N^{ϵ}-(DL-2-amino-2-carboxyethyl)-L-lysine; LAL), a recently recognized amino acid that has been found to occur as a protein-bound residue in various alkali-treated edible and non-food proteins, has also been found in many food proteins which have been subjected to conventional home cooking procedures under non-alkaline conditions. Rats fed diets containing freely available LAL, either in the form of a hydrolysed alkali-treated protein or as the free synthetic amino acid, exhibit a unique microscopic lesion of the kidney, characterized by nuclear and cytoplasmic enlargement (cytomegaly) of epithelial cells in the straight portion of the proximal tubules. Such findings have prompted some to suggest that alkali-treatment of protein might have important toxicological implications for man. Failure to induce the renal cytomegalic lesion in six other species by feeding free LAL at levels greatly in excess of that which is uniformly nephrotoxic to the rat (100 ppm) strongly suggests that LAL-induced cytomegaly is a phenomenon specific to the rat species.

Lysinoalanine (LAL) in alkali-treated proteins

Alkaline treatment of food proteins was known to primitive cultures long before recorded time and is now firmly established in modern food technology for the making of protein isolates, for the production of functional proteins with desired foaming, emulsifying and stabilizing characteristics, and for the preparation of solutions suitable for the spinning of protein fibre (Circle & Smith, 1972).

Recently, Woodard & Alvarez (1967) and Woodard (1969) described the occurrence of a unique histological lesion in the kidneys of rats fed an industrial-grade soya protein and attributed it to the formation of a toxic factor in the protein as a result of alkali treatment. Subsequently, this group reported similar lesions, termed nephrocytomegalia, in the kidneys of rats fed an edible-grade soya protein, which had been subjected to harsh alkali treatment (Woodard & Short, 1973). These studies appear to have been prompted by an earlier study reported by Newberne & Young (1966), who first observed this peculiar lesion in rats fed an industrial-grade soya protein. However, as other investigators (de Groot & Slump, 1969; van Beek, Feron & de Groot, 1974) failed to reproduce the lesion under the conditions described by Woodard and his colleagues, it is pertinent to review the matter in some detail.

In seeking to elucidate the effects in weanling rats of a protein that was marginally or grossly deficient in sulphur-containing amino acids and Vitamin B_{12}, Newberne & Young (1966) chose for their nutritional studies an industrial grade of soya protein known as Alpha Protein (a product of Central Soya Co., Chicago, Ill.). Alpha Protein is a soya-protein product which has been subjected to severe caustic treatment, permitting sufficient hydrolysis to elicit the various adhesive and viscid properties required in the manufacture of some water-based paints and in the pigment-coating of paper.

Alpha Protein was never intended nor produced for food use, but Newberne & Young (1966) found it useful in their studies because of its low methionine content and lack of cystine. They noted that Sprague–Dawley-derived Charles River rats fed a semi-synthetic diet containing 20% Alpha Protein, low in methionine and choline, exhibited marked nuclear and cytoplasmic enlargement of the epithelium of the pars recta (straight portion) of the proximal tubules in the region of the cortico-medullary junction of the kidney. These histological changes, unattended by clinical evidence of disease, occurred in 87–90% of the rats fed the diets most deficient in methionine and choline. Supplementation of the low-methionine diet with 0·3% choline reduced the incidence of renal alterations slightly, to 78%. Administration of a diet low in choline, but with moderate methionine supplementation, greatly reduced the incidence of these renal lesions, with only approximately 16% of animals showing cytomegalic changes. This incidence was further decreased significantly to 7% in rats fed a combined high-methionine (0·6%)–high-choline (0·6%) supplement. With the addition of vitamin B_{12} to this latter regimen, histomorphology of the kidney was found to be completely normal. This study suggested that the renal cytomegalic lesion was the result of a multiple nutritional defect involving methionine, choline and vitamin B_{12}, although the authors did not overlook the possibility that a toxic factor might be involved.

Subsequently, Woodard & Alvarez (1967) and Woodard (1969) reported the occurrence of renal cytomegaly in Sprague–Dawley rats fed diets containing 20–30% Alpha Protein. Further, it was noted that

the supplementation of the Alpha-Protein diet with methionine, choline and vitamin B_{12} to levels well above those required for optimum growth failed to prevent the development of the renal lesions. The view was put forward that the industrial protein* contained a toxic substance that was responsible for the renal changes and the authors "wondered" whether a similar substance might exist in alkali-treated proteins used in food products for human consumption (Woodard & Alvarez, 1967). It was also noted that, while the rats fed Alpha Protein showed some depression of growth rates compared with the controls, they exhibited neither clinical evidence of illness nor an increase in mortality in association with the reported renal lesions. Rats fed an edible grade of soya protein, Promine D® (Central Soya Co., Chicago, Ill.) did not develop renal cytomegaly (Woodard, 1969).

Meanwhile, de Groot & Slump (1969) of the Dutch Central Institute for Nutrition and Food Research TNO (CIVO) reported the occurrence of a previously unrecognized amino acid in several alkali-treated food proteins of animal and vegetable origin. This amino acid, which has been given the trivial name lysinoalanine (LAL) and is formally designated N^{ϵ}-(DL-2-amino-2-carboxyethyl)-L-lysine, had first been discovered independently by Bohak (1964) and Patchornik & Sokolovsky (1964) following alkaline treatment of bovine ribonuclease. LAL was shown to be formed during the alkaline treatment of some proteins by the addition of the ϵ-amino group of a lysyl residue to the double bond of a dehydroalanyl residue formed by a β-elimination reaction from cystine or serine.

The CIVO group had found progressively increased LAL formation in alkali-treated soya protein as pH, temperature and reaction time were increased. They noted that, under extreme conditions, net protein utilization and digestibility of the treated protein were reduced. However, when a treated protein representative of the severely alkalinized preparations (pH 12·2 at 40°C for 4 hours) was fed to young rats for 90 days, it failed to produce any evidence of renal cytomegalic changes despite the fact that the CIVO diet contained LAL levels similar to those of the diets used by Woodard (1969) and Woodard & Alvarez (1967), i.e. 0·12%. No clinical or histological abnormalities were observed other than an increased degree of nephrocalcinosis in the females, a condition which is readily alleviated by adjustment of the level of phosphorus and calcium in the diet (de Groot & Slump, 1969).

*It should be noted that Alpha Protein was batch-produced in at least three viscosity grades (low, medium and high) to satisfy a variety of industrial applications, and protein hydrolysis is likely to have occurred to a varied degree. Understandably, batch-to-batch tolerances for such materials were not rigid. In more recent years, automated manufacturing procedures have been introduced, permitting somewhat narrower production tolerances. While it is not known with certainty, it is believed that low-viscosity grades of Alpha Protein were used in these studies. The manufacturers ceased commercial production of Alpha Protein in the spring of 1975 (E. W. Meyer, Central Soya Co., Chicago, personal communication 1975).

Subsequently, Woodard (1972) and Woodard & Short (1973) reported the induction of renal cytomegaly in Sprague–Dawley rats fed the edible-grade soya protein, Promine D, which had been subjected to severe alkali treatment (0·1 N-NaOH at 60°C for 8 hours). Further, they proposed that the toxic factor in Alpha Protein and in alkali-treated Promine D responsible for the renal cytomegalic lesion was LAL. In response to the suggestion (Woodard & Short, 1973) that the Dutch investigators may have failed to recognize the renal lesion, Dr. Woodard was supplied with slides of the kidneys from the rats used in the original Dutch study and has since agreed with the Dutch pathologists that none of the cytomegalic changes he described were to be found in these animals (A. P. de Groot, personal communication 1973).

To resolve these discrepant observations, the Central Institute for Nutrition and Food Research TNO (CIVO) undertook in-depth studies to permit the full assessment of the safety of alkali-treated food-grade spun soya protein and to examine the conditions necessary for the exhibition of LAL-related nephrotoxicity.

Studies of alkali-treated spun soya protein in rats

Spun soya isolate, an alkali-treated edible soya protein used in the production of meat analogues, was incorporated in test diets at levels of 5, 10 and 20%, replacing equivalent amounts of protein from untreated soya isolate. Two control diets were used, one containing 20% untreated soya protein isolate, the other containing 20% casein protein. The test and control diets were fed to Wistar-derived rats of the CIVO colony for 13 weeks. Except for a relative increase in kidney weights and a degree of nephrocalcinosis in the females, findings that were related to the dose level of spun soya isolate and the amount of available KH_2PO_4 added to the test diets, no adverse effects were noted with respect to physical appearance, growth, feed efficiency, haematological and blood-serum values, renal function or organ weights or upon gross and histological examination. Specifically, careful histological study of the kidneys did not reveal any evidence of cytomegalic alteration of the tubules of the pars recta (van Beek *et al.* 1974). The LAL contents of the test diets (ranging from 0·01 to 0·041%) were considerably less than that (0·12%) in the diets used by Woodard & Short (1973), but it should be noted that de Groot & Slump (1969) had earlier failed to observe renal cytomegaly in rats fed diets containing LAL at levels of the order of 0·12%.

Studies of Alpha Protein in rats

In a corollary study (van Beek *et al.* 1974), attempts were made to reproduce the renal tubular cell changes described by Woodard and his colleagues by feeding rats Alpha Protein at the 20% level, after incorporation in either the CIVO stock diet or in a diet similar in composition to that used by Woodard & Short (1973). Nephrocytomegaly was not found in any of the rats fed either of the test diets. The rats fed the simulated Woodard diet suffered diarrhoea and failed to show optimum physical condition

and it was noted that they exhibited distinct growth retardation, lower food intake and reduced food efficiency. Amino acid supplementation studies suggested that the inferiority of the Woodard-type diet was due primarily to a deficiency of methionine and threonine (van Beek *et al.* 1974).

The Woodard feeding studies involved the use of Sprague–Dawley-derived Charles River rats, while those of the Dutch investigators were carried out on Wistar rats of the CIVO colony. To rule out strain differences as a reason for the contradictory findings of the two groups, a subsequent study involved the feeding of Alpha Protein to three separate strains of weanling rats (de Groot *et al.* 1976a,b). Two different Wistar-derived strains and one Sprague–Dawley strain received test diets containing either 20% Alpha Protein, 20% Alpha Protein plus 10% casein or 35% Alpha Protein. The 20%-Alpha Protein diets provided LAL levels of 0·07%, while the 35%-Alpha Protein regimen provided 0·12% LAL. Control groups were fed a 33%-casein diet. Animals were killed for study after 4 and 12 weeks of feeding. The 20%-Alpha Protein diet supported growth very poorly in all three strains, while the diet of 20% Alpha Protein plus 10% casein afforded much improved growth. The 35%-Alpha Protein regimen permitted further improvement in the rate of growth, which was comparable to, but somewhat less than, that achieved with the 33%-casein diet. Feeding of Alpha Protein at levels providing 0·07% or 0·12% LAL in the diet failed to produce renal cytomegaly in the three strains of rat examined. The considerable degree of improvement in growth and food efficiency achieved by increasing the dietary level of Alpha Protein from 20 to 35% was not in keeping with the idea that the protein is toxic.

Observations with free synthetic LAL

Synthetic LAL was prepared as the dihydrochloride from lysine according to a modification of the method of Okuda & Zahn (1965) by the Organic Synthesis section of The Central Institute for Nutrition and Food Research TNO (CIVO). The resulting LAL.2HCl, which contained 0·8% lysine as its major contaminant, was fed to rats in a short-term (28-day) study at levels of 0·0, 0·1, 0·3 and 1·0% of the CIVO stock diet. While gross autopsy findings were negative, microscopic examination of the kidneys in all the test groups revealed a severe tubular lesion restricted to the cortico-medullary junction and consisting of necrosis, regeneration and cytomegaly of the epithelial cells of the straight portion (pars recta) of the proximal tubules. The cytomegaly was characterized by enlarged cells which contained gigantic vesicular nuclei, often containing multiple nucleoli and exhibiting a reticular chromatin pattern.

Additional 28-day feeding studies, directed towards establishing a threshold effect level, revealed the consistent induction of nephrocytomegaly in rats fed a diet to which 0·01% synthetic LAL had been added, while no nephrotoxic effect was observed when free LAL was added to the diet at a level of 0·003%.

It was concluded from these studies that free synthetic lysinoalanine is nephrotoxic to rats (de Groot *et al.* 1976a).

Comparative effects in rats of synthetic LAL and alkali-treated soya protein, before and after complete acid hydrolysis

This study was prompted by the observation that free synthetic LAL will induce renal cytomegalic alterations in all weanling rats when fed for 4 weeks at a dietary level of 0·01%, yet no such lesions were observed in rats fed Alpha Protein containing 0·12% LAL. These findings suggested a distinct difference in the nephrotoxic potential of free and protein-bound LAL. To test this hypothesis, seven groups of male weanling rats were fed the following soya-protein preparations, as 20% of the diets, for 28 days: soya-protein isolate (ISP; negative control), acid-hydrolysed soya-protein isolate (ISPH), alkali-treated soya-protein isolate (ISPA), acid-hydrolysed alkali-treated soya-protein isolate (ISPAH) and ISP plus 0·02, 0·06 or 0·18% added synthetic LAL (positive controls). The alkali-treated proteins (ISPA and ISPAH) had been subjected to 0·1 N-NaOH at 60°C for 8 hours and had subsequently been precipitated with acid, as described by Woodard & Short (1973). The calculated LAL content of the ISPA and ISPAH diets was 0·18 g/100 g diet as dry matter. Acid hydrolysis was accomplished by refluxing ISP and ISPA with 6 N-HCl for 22 hours. Diets containing 20% ISP plus 0·02, 0·06 or 0·18% synthetic LAL served as positive controls. Dietary LAL content and test results in terms of growth effect, efficiency and nephrocytotoxicity are given in Table 1.

Table 1. *Effects of protein modification in terms of growth response, feed efficiency and renal cytotoxicity**

Diet	Growth depression	Reduced feed efficiency	Nephro-cytomegaly	LAL in diet (%)
ISP	−	−	−	
ISPH	+	−	−	
ISPA	−	−	−	0·24
ISPAH	+	+	+	0·17
ISP + 0·02% LAL	−	−	+	0·02
0·06% LAL	−	−	+	0·06
0·18% LAL	+	+	+	0·18

LAL = Lysinoalanine ISP = Soya-protein isolate ISPH = Acid-hydrolysed ISP
ISPA = Alkali-treated ISP ISPAH = Acid-hydrolysed alkali-treated ISP
*Data kindly furnished by Drs A. P. de Groot, V. J. Feron and P. Slump.

TABLE 2. *LAL content in serum, urine and faeces of five male rats fed ISPA or ISPAH**

Dietary protein source	Amount of LAL consumed (mg)			Amount of LAL excreted (mg)		Serum LAL† (μg/ml)
	Free	Protein-bound	Total	Urine†	Faeces†	
ISPA	—	268	268	0·6	135·1	0·4
ISPAH	140	—	140	34·4	9·6	2·3

LAL = Lysinoalanine ISPA = Alkali-treated soya-protein isolate
ISPAH = Acid-hydrolysed alkali-treated soya-protein isolate
*Data kindly supplied by Drs A. P. de Groot, P. Slump, V. J. Feron and L. van Beek.
†Determined as free LAL in serum and urine; faecal LAL determined after hydrolysis; LAL content of hydrolysed urine from ISPA-fed rats was three times higher than that of unhydrolysed urine.

Growth rate depression was seen in the rats fed ISPH, ISPAH and ISP + 0·18% LAL. Growth retardation of the ISPH group was attributed to unpalatability of the diet since feed efficiency was not affected, but growth-rate depression in the groups fed ISPAH and ISP + 0·18% LAL was accompanied by reduced feed efficiency. Severe tubular alterations in the region of the cortico-medullary junction, characterized by necrosis, regeneration and cytomegaly of the proximal tubular epithelium were noted in all rats fed ISPAH or LAL. In contrast, the feeding of ISPA, affording a level of 0·18% protein-bound LAL, was not associated with growth retardation, reduced feed efficiency or nephrocytomegaly. Thus, it may be concluded that freely available LAL is definitely nephrocytotoxic to rats (de Groot *et al.* 1976b). This conclusion is further supported by the results of LAL analyses of blood, urine and faeces, which show that the degree of absorption of LAL in ISPA is considerably lower than that of LAL in ISPAH (Table 2).

Studies of oligopeptide-bound LAL

In their original report, de Groot & Slump (1969) had noted that a commercial modified-protein product (CP), a foaming agent used in confectionery manufacture, was found to have a total LAL content of 5·5 g/16 g N, equivalent to 3·18 g/100 g product. Upon further analysis it was found that CP consisted of partially hydrolysed protein with a large amount of LAL bound in soluble oligopeptides. Molecular-weight distribution of the soluble oligopeptide-linked LAL fractions was determined after paper filtration of the aqueous suspension at pH 3·5 and subsequent separation over polyacrylamide gels (Bio-Gel P-2 and P-6, 200–400 mesh), as well as after trichloroacetic acid (TCA) precipitation. As shown in Table 3, CP was found to contain 22,900 ppm LAL (72% of the total amount of LAL) in a form that was soluble in water at pH 3·5 and 7400 ppm in a form soluble in an aqueous solution of 10% TCA. The free LAL content was only 26 ppm.

Having demonstrated that the feeding of protein-bound LAL in high concentration did not damage the rat kidney, while free LAL at dietary concentrations of 0·01% was uniformly nephrotoxic, the Dutch investigators were prompted to study the nephrocytotoxic potential of oligopeptide-bound LAL present in the CP.

The CP was incorporated at a level of 15% in test diets with either 10 or 20% casein, to provide approximately 5500 ppm LAL. A 20%-casein diet served as the control. Dietary and analytical data, kindly supplied by Dr. P. Slump, are presented in Table 4.

Each of the diets was fed to a group of ten male weanling rats from the CIVO colony for 4 and 8 weeks. Examination of the kidneys of rats fed the CP diets for 4 weeks failed to reveal the cytomegalic

Table 3. *Molecular-weight distribution of oligopeptide-linked LAL in modified protein**

LAL fraction	LAL content of CP† (ppm in CP)	
	Extracted with water at pH 3·5	Extracted with 10% aqueous TCA
Peptide-linked plus free LAL	22,900	7400
Molecular weight distribution		
>5000	5200 (23%)	
5000–1500	13,100 (57%)	
1500–1000	2100 (9%)	
1000–500	1400 (6%)	
500	1100 (5%)	
Free LAL	26	26

LAL = Lysinoalanine CP = Modified protein
*Data by courtesy of Dr. P. Slump.
†Total amount of LAL present in CP was 31,800 ppm.

TABLE 4. *Percentage composition of modified casein diets**

Ingredient	Composition (%) of diet fed to group no.		
	7477	7478	7479
Components			
Casein	20	10	20
Test protein CP	—	15	15
Wheat starch	41	37	27
Sucrose	20	20	20
Cellulose	6	6	6
Mineral mixture	4	4	4
$CaCO_3$	1	—	—
Vitamin ADEK preparation	0·4	0·4	0·4
Vitamin B mixture	0·2	0·2	0·2
Choline chloride (50%)	0·4	0·4	0·4
Soya-bean oil	7	7	7
By analysis			
Crude protein (N × 6·25)	17·6	18·4	27·9
Calcium	1·04	2·08	2·07
Phosphorus	0·53	0·54	0·62
LAL	—	0·55	0·55
Calculated			
LAL: insoluble, protein- or peptide-linked	—	0·21	0·21
soluble, peptide-linked	—	0·34	0·34
soluble, peptide-linked (mol wt < 1500)	—	0·07	0·07
soluble, peptide-linked (mol wt > 5000)	—	0·08	0·08
free	—	0·0004	0·0004

CP = Modified casein LAL = Lysinoalanine

*Data by courtesy of Dr. P. Slump.

lesion, although slight tubular dilatation and an occasional enlarged tubular epithelial cell were seen in the cortico-medullary area. In contrast, rats fed the CP diets for 8 weeks showed slight but distinct nephrocytomegaly and this was more pronounced in the rats on the 15% CP + 10% casein diet than in those on the 15% CP + 20% casein diet. These diets provided 2100 ppm insoluble protein-bound LAL and 3400 ppm soluble peptide-linked LAL. The latter fraction contained 800 ppm of mol wt > 5000, 1900 ppm of mol wt between 5000 and 1500, 700 ppm of mol wt < 1500 and only 4 ppm as free LAL. Since the level of free LAL in the CP diets was 25 times lower than the lowest effect level (100 ppm) of synthetic LAL, the occurrence of nephrocytomegaly in this study cannot be ascribed completely to free LAL. While the lesions induced by the CP diets were much less pronounced than those associated with diets containing 100 ppm synthetic LAL, it appears that one or more peptides containing LAL are active in terms of inducing renal cytomegaly in rats (de Groot *et al.* 1976b). Obviously, oligopeptide-bound LAL is active in this respect. Fully comparable results have been obtained from a recent model study with a commercial acid-precipitated casein which was treated with alkali to produce a content of LAL in excess of 5%, followed by partial acid hydrolysis (de Groot *et al.* 1976b).

Absence of LAL-induced renal cytomegaly in species other than the rat

de Groot *et al.* (1974, 1976a,b) have demonstrated than weanling rats are extremely sensitive to the renal cytotoxic effect of freely available dietary LAL. A unique lesion characterized by cytoplasmic and nuclear enlargement of the epithelium of the pars recta of the proximal tubules is readily induced in rats fed synthetic LAL at a dietary level of 100 ppm for 4 weeks, yet no such cytotoxic effect is apparent when free LAL is fed at a level of 30 ppm. Earlier, the contradictory findings of the American and Dutch groups on the feeding of Alpha Protein and an edible-grade protein which had been subjected to severe alkali treatment, prompted studies to show that strain difference among rats was not a factor. It was then necessary to determine whether other animal species were susceptible to LAL-induced nephrocytomegaly.

Swiss mice, Golden Syrian hamsters, New Zealand white rabbits and Japanese quail were fed diets supplemented with 1000 ppm synthetic LAL without untoward effect on growth rate, food intake or food efficiency, while histological examination of the kidneys after the feeding of these diets for 4 and 8 weeks failed to reveal renal cytomegaly or other evidence of visceral toxicity (de Groot *et al.* 1976a). Two pure-bred male Beagle pups, aged 6 months, fed a diet supplemented with 700 ppm synthetic LAL, one for 4 weeks and one for 9 weeks, failed to show any histological evidence of nephrocytomegaly or other visceral pathology, and growth-rate and food-intake patterns were within the normal range (de Groot *et al.* 1976a). Male rhesus monkeys were fed diets containing either 1000 ppm free synthetic LAL or 10,000 ppm protein-bound LAL, provided by alkali-treated casein, for a period of 8 weeks without evidence of adverse effect on growth; neither renal cytomegaly nor other evidence of visceral pathology related to treatment was observed on microscopic examination (de Groot *et al.* 1976b). Thus, it appears that of the seven animal species studied so far, only the rat is responsive to the renal cytotoxic effect of freely available LAL.

Historical note on alkali treatment of foods

In a fascinating anthropological study, Katz, Hediger & Valleroy (1974) have noted that the development of maize (Indian corn) as a cultigen from wild precursors indigenous to the Western Hemisphere in the period 3000–3500 BC was one of the three major agricultural revolutions in the past 10,000 years and made an important contribution to the rise of the great Mesoamerican civilizations. Even today, corn, which is the largest single crop in the United States, remains the chief source of calories and protein for most inhabitants of Latin America (Wilkes, 1972). The use of alkali treatment was critical to the successful exploitation of corn as a protein source in order to render available the limited lysine and tryptophan content from the glutelin fractions, which are normally indigestible by man and other non-ruminant creatures. Geographical location determined whether the source of the alkali was lime, yielding $Ca(OH)_2$ in solution, wood ashes with their yield of KOH, or lye, to give NaOH (Katz *et al.* 1974).

Tortillas, the basic staple of diets in Mexico and Central America (Bressani, Paz y Paz & Scrimshaw, 1958; Bressani & Scrimshaw, 1958) are prepared by heating whole corn in 0·5–1·0% lime solution at 80–94°C for 25–60 minutes and allowing the mixture to cool for about 14 hours. The supernatant is then removed, and the corn is washed and drained for milling into a dough or 'masa' for subsequent baking in the form of cakes. While such treatment has been shown to decrease the overall nutrient content of the corn, Bressani & Scrimshaw (1958) and others (Cravioto, Anderson, Lockhart, Miranda & Harris, 1945) have demonstrated improvement in the overall nutritional quality of corn by alkali treatment. As the essential amino acid, tryptophan, is the precursor in the endogenous formation of the B vitamin, niacin, one can appreciate the importance of its availability in a society in which the principal protein-calorie staple is corn. In fact, it has been suggested that pellagra, or niacin deficiency, which was widely prevalent in the southern United States during the depression of the 1930s, may well have been due to the extensive consumption of corn grits which had not been alkali-treated (Katz *et al.* 1974).

Ubiquity of LAL in alkali- and/or heat-treated proteins

LAL formation has been demonstrated in many alkali-treated edible and non-food proteins, such as ribonuclease A, phosvitin, bovine plasma albumin, papain and lysozyme (Bohak, 1964), wool (Asquith & Garcia-Dominguez, 1968; Miro & Garcia-Dominguez, 1967; Ziegler, 1964), soya protein, casein, groundnut meal, sesame protein and brewers' yeast protein (de Groot & Slump, 1969), bovine cartilage (Whiting, 1971) and sunflower protein (Provansal, Cuq & Cheftel, 1975). Its formation is enhanced under conditions of increasing pH, temperature and reaction time.

In view of the important role of alkali treatment in the successful exploitation of protein sources throughout history and its many applications in modern food technology, Sternberg, Kim & Schwende (1975b) were curious to learn the extent to which LAL might be found in our everyday diet. Samples of industrially processed food ingredients and prepared foods, as well as meats and poultry products prepared under home-cooking conditions, were analysed for LAL content after hydrolysis in 6 N-HCl (Sternberg, Kim & Plunkett, 1975a). Seven commercial samples of sodium and calcium caseinate were found to contain from 250 to 6900 ppm LAL (μg/g protein), while three preparations of acid casein contained 70–190 ppm. Four preparations of dried egg-white solids contained between 160 and 1820 ppm LAL. Brand-named corn chips, pretzels and hominy ranged in LAL content from 390 to 560 ppm. Three standard brands of evaporated milk and two of condensed milk had LAL contents of 360–860 ppm. Six commercial infant milk formulas, soya and casein-derived, contained from 150 to 640 ppm LAL. Commercial preparations of tortillas and masa assayed at 200–480 ppm LAL.

It was noted with particular interest that LAL is formed in appreciable amounts in many food proteins when they are heated in non-alkaline conditions. Using conventional home-cooking procedures (including boiling, frying and broiling), the LAL content of frankfurters varied from 50 to 150 ppm. Egg-white yielded 140 ppm LAL after being boiled for 3 minutes, while the pan scrapings from a fried sirloin steak contained 130 ppm LAL. When soya globulin, ovalbumin, casein, lysozyme and bovine serum albumin, all of which are found naturally in food, were heated in the absence of alkali, LAL was formed in all samples under the conditions of pH, temperature and time commonly used in conventional home-cooking and commercial-processing procedures (Sternberg *et al.* 1975b).

Discussion

That LAL should be regarded as a new and unusual amino acid can be justified only by virtue of its recent discovery in a variety of edible and non-food proteins. LAL must long have been a component of man's food. In noting that the development of maize as a primary cultigen constituted one of the three major agricultural revolutions in the last 10,000 years, Katz *et al.* (1974) emphasized that the application of alkali-processing techniques was critical to its successful exploitation as a major source of protein nutrition among the Mesoamerican civilizations. Sternberg *et al.* (1975b) have shown that LAL may be generated even in the absence of alkaline conditions, by the application of conventional home-cooking procedures, in such foods as egg-white, chicken, steak and frankfurters. In fact, LAL has been found to develop spontaneously in freshly laid eggs after storage for a few days (de Groot *et al.* 1976a). Certainly, we can assume that LAL has been a component of dietary protein since man first learned to cook.

On the basis of the finding of a unique histological abnormality, termed cytomegaly, in the kidneys of rats fed either a partially hydrolysed alkali-treated industrial protein or a soya protein that had been subjected to severe and prolonged alkali treatment at elevated temperatures, Woodard & Short (1973) have implied that the use of alkali-processing techniques

employed in the food industry may have important toxicological implications. To this reviewer's knowledge, the nephrocytomegalic lesion described by Woodard & Alvarez (1967) and by Woodard (1969) has not been demonstrated to occur in man as a consequence of the ingestion of alkali-treated protein.

de Groot *et al.* (1976a,b) have shown that rats are extremely sensitive to the nephrocytomegalic effects of LAL supplied in the diet as the free synthetic amino acid or rendered freely available from an alkali-treated protein by complete acid hydrolysis. The dietary addition of 100 ppm free synthetic LAL was consistently nephrocytotoxic to rats, while a dietary level of 30 ppm was without effect. On the other hand, feeding of an alkali-treated protein providing 2400 ppm protein-bound LAL was devoid of renal cytomegalic effect, while the same protein provided in the diet as a complete acid hydrolysate induced marked cytomegaly in the cortico-medullary zone, accompanied by necrosis and regeneration of the tubular epithelium. Diets containing a partially acid-hydrolysed alkali-treated protein providing as much as 5500 ppm LAL, principally in soluble polypeptide- and oligopeptide-linked form, failed to induce the renal abnormality in rats fed for 4 weeks, but produced a distinct, although mild, cytomegalic lesion after 8 weeks of feeding.

It will be recalled that while the renal cytomegalic lesion was first recognized in rats fed Alpha Protein (Newberne & Young, 1966; Woodard & Alvarez, 1967), de Groot *et al.* (1976a) were unable to induce the lesions in rats fed a recently obtained supply of Alpha Protein at levels providing 700 and 1200 ppm LAL. As strain differences in rats were ruled out in an effort to explain these contradictory findings, it is strongly suspected that the Alpha Protein used in the American experiments was batch-produced material which had undergone more severe hydrolysis than the product supplied to the Dutch group and made under closely monitored, automated production procedures.

In marked contrast to the findings noted in the rat, which is consistently sensitive to the renal cytotoxic effects of as little as 100 ppm free LAL, de Groot *et al.* (1976a,b) were unable to induce the renal lesion in dogs fed 700 ppm synthetic LAL or in quail, mice, hamsters, rabbits or monkeys fed with a level of 1000 ppm free LAL. Thus there is strong evidence that LAL sensitivity is specific to the rat, a phenomenon that should give investigators reason to pause before extrapolating new and unusual toxicological observations from a single species to man.

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Fd Cosmet. Toxicol. Vol. 14, pp. 491–492. Pergamon Press 1976. Printed in Great Britain

REVIEWS OF RECENT PUBLICATIONS

Survey of Lead in Food: First Supplementary Report. Working Party on the Monitoring of Foodstuffs for Heavy Metals: Fifth Report. Ministry of Agriculture, Fisheries and Food. HMSO, London, 1975. pp. 34. £0.50.

Since the first report on this subject was published 4 years ago (*Cited in F.C.T.* 1973, **11,** 119), there appears to have been a welcome decline in the lead content of the British diet. During 1972 and 1974, analysis of 397 samples, representing each of the eight major food groups, indicated that the average lead content of the total diet had fallen to 0·09 ppm from the previous value of 0·13 ppm. This decline was particularly evident in root vegetables and cereals, and only in fish did there appear to be a slight increase (from 0·08 to 0·11 ppm). The weekly intake of lead from the 1·5 kg of food consumed daily by the average person was calculated as approximately 1 mg, and a further 0·2 mg/week was found to be derived on average from a 1 litre/day consumption of drinking-water. The average total weekly intake of 1·2 mg lead was thus well below the 3 mg regarded by the Joint FAO/WHO Expert Committee on Food Additives as "provisionally tolerable" (*ibid* 1973, **11,** 655; *ibid* 1974, **12,** 407).

Of some 6000 individual food items analysed, very few were found to exceed the statutory limits laid down in the Lead in Food Regulations 1961, the only exceptions being herbs, liver and certain canned foods. In the first two categories, values ranged up to 16·0 and 7·30 ppm, respectively, but contamination of herbs was thought to have resulted from incomplete removal of soil particles, and of 335 samples of liver, the lead in only 13 exceeded 2 ppm. The canned foods involved were mainly acid fruits packed in lacquered cans, rather than plain cans in which the tin acts as a sacrificial anode (*ibid* 1976, **14,** 340) but confidence is expressed that continuing progress in canning technology will eliminate this problem. In canned baby foods, mean levels had declined from the 0·24 ppm recorded in the 1972 report to 0·07 ppm, the latter being very similar to the 0·08 ppm found in baby foods in jars and well below the maximum of 0·5 ppm prescribed by current regulations (Lead in Food (Amendment) Regulations 1972; Statutory Instrument 1972, no. 1843).

In fish landed in England and Wales the average lead content was below 0·7 ppm; when weighted according to the relative proportions of different fish species consumed, this value fell to 0·1 ppm. Shellfish, excluding crab, contained a weighted average level of 1·13 ppm; although samples taken from certain polluted areas such as the Severn estuary contained much higher levels (up to 8·4 ppm in winkles, for example), no major commercial fisheries occur in these areas. Crabs were generally less contaminated, containing on average less than 0·3 ppm, even in the brown meat. Vegetables grown in areas where the natural lead content of the soil is high, near smelters or on land treated with sewage sludge contained higher-than-average lead levels, but this was thought to be due partly to contamination with soil particles, which would be removed during preparation and cooking.

Of nearly 11,000 samples of drinking-water, only 0·2% exceeded the WHO recommended limit of 0·1 mg/litre, the exceptions being typically from houses built more than 20 years ago with lead pipes in areas of plumbosolvent water. The extent of this problem is now being assessed in a nationwide survey by the Department of the Environment, and possible means of reducing levels are being given urgent consideration.

In an appendix to the report, the Toxicity Sub-Committee concludes that the present dietary intake of lead is unlikely to constitute a hazard to the general population. Studies about to be undertaken in selected areas are thought likely to be of considerable value in assessing maximal intakes and in delineating problem areas, and information on the dietary lead intake in children of different age groups is also regarded as necessary. It is considered that efforts should be made to reduce lead in those canned foods which contain relatively high levels, and to identify the source of lead in fresh foods with a high lead content. Inadequate information is as yet available on the lead content of some foodstuffs, and it is recommended that monitoring should be continued. In a second appendix these conclusions are endorsed by the Food Additives and Contaminants Committee, which places special emphasis on the need for eliminating lead from canned foods and for monitoring lead consumption by children and high-intake groups.

Although these findings are fairly reassuring for the great majority of the population, it is disturbing to see that a single meal composed of a shellfish *hors-d'oeuvre*, followed by liver and by a dessert concocted of canned fruit such as loganberries, might well expose the consumer to more than the 3 mg lead considered "provisionally tolerable" as a total weekly intake. In this connexion the survey of blood lead levels in individuals with a high shellfish intake, recommended in the previous report but apparently not yet undertaken, would be of particular value. Advances in canning technology will solve some problems, but naturally-occurring lead in fresh foods such as shellfish could possibly be reduced only by far-reaching controls over industrial water pollution and/or by as yet undeveloped processing techniques.

Pesticide Residues in Food. Report of the 1974 Joint Meeting of the FAO Working Party of Experts on Pesticide Residues and the WHO Expert Committee on Pesticide Residues. *Tech. Rep. Ser. Wld Hlth Org.* 1975, **574.** pp. 37. Sw.fr. 6.00 (available in UK through HMSO).

1974 Evaluation of Some Pesticide Residues in Food. The Monographs. WHO Pesticide Residues Series, No. 4. WHO, Geneva, 1975. pp. 545. Sw.fr. 48.00 (available in UK through HMSO).

A joint meeting of the FAO Working Party of Experts on Pesticide Residues and the WHO Expert Committee on Pesticide Residues was held in Rome in December 1974.

In the report of the meeting and the accompanying monographs 35 pesticides are considered. The following acceptable daily intake values (ADIs) were allocated (in mg/kg body weight): amitrole, 0·00003 (conditional); chinomethionat, 0·003T (temporary); chlorothalonil, 0·03T; dichlofluanid, 0·3T; 2,6-dichloro-4-nitrobenzenamine, 0·03T; dodine, 0·01T; fenamiphos, 0·0006, pirimiphos-methyl, 0·005T; fenitrothion, 0·005; 2,4-D, 0·3; dimethyl dithiocarbamates, 0·005T; ethylene bis-dithiocarbamates, 0·005T. The data for daminozide, dinocap and tecnazene, although evaluated, were considered insufficient for the allocation of ADIs.

The first ten of the pesticides listed above were also allocated new or revised maximum residue limit values. In the absence of an ADI it was thought impossible to recommend limits for leptophos, but guidelines indicating the level of residues resulting from recommended uses of this insecticide were published for the information of regulatory authorities.

The report draws attention to the allocation of ADIs to amitrole and the dithiocarbamates. Both amitrole and ethylene thiourea, a degradation product of the ethylene bis-dithiocarbamates, have produced thyroid tumours in mice and rats. It was suggested that this tumour formation was due to the toxic antithyroid action of the compounds. In both cases no-effect levels for biochemical and histochemical changes in the thyroid gland have been demonstrated, and in view of this the meeting felt justified in allocating ADIs (with qualifications) for amitrole and the dithiocarbamates.

The ability of certain nitrogen-containing pesticides to form *N*-nitroso compounds *in vivo* and *in vitro* was discussed briefly. The meeting recommended that further work be carried out on the formation of nitrosamines from pesticides under conditions and in concentrations that included those to which man might be exposed.

The situation regarding two organochlorine pesticides, DDT and hexachlorobenzene (HCB), was again reviewed. The meeting considered new data which demonstrated that mice ingesting 36 mg DDT/kg for 15 weeks developed persistent hepatomas. However, it was stressed that no similar tumours had been produced in any other species tested and that the epidemiological evidence gave no indication that DDT was a human carcinogen. The meeting considered that there was an urgent need for new epidemiological data that would permit an evaluation of the implications of the experiments in mice.

HCB was reviewed in the light of the results of a number of new short-term studies. A final decision on an ADI was deferred pending the results of two long-term studies currently in progress, but a conditional ADI of 0·0006 mg/kg was assigned. Some concern was noted over the increased number of reports of HCB residues in foods, feeds and human tissues.

In a more general vein, the purity of pesticides used in toxicological evaluations was discussed; although the evaluations are normally carried out using technical-grade commercial material, the meeting was anxious to avoid complications due to the presence of potentially toxic impurities. It was suggested that, in future, FAO/WHO monographs on pesticides should include some details of industrial synthesis. This would help in forecasting the possibility that a toxicologically significant impurity might be formed during manufacture.

Fd Cosmet. Toxicol. Vol. 14, pp. 493–496. Pergamon Press 1976. Printed in Great Britain

BOOK REVIEWS

Food Protein Sources. International Biological Programme 4. Edited by N. W. Pirie. Cambridge University Press, London, 1975. pp. xx + 260. £7.50.

This book is one of a series summarizing the results of national and international studies carried out within the framework of the International Biological Programme, which was established to meet the need for a better understanding of man's environment in order to provide for the rational management of natural resources. Volume 4 draws together contributions describing the major types of protein source, and divides them clearly into three main classes: sources edible after minimal processing, concentrates made by mechanical extraction and concentrates made by biological conversion. The lower limit for a 'protein source' has been set arbitrarily at 15% protein. This definition excludes those cereals that supply more than half the protein now eaten, but not some of the newer cereals.

Within the first class of protein source considered are protein-rich cereal seeds, seed legumes, some minor food seeds, vegetables and algae. The second class includes coconuts, soya beans, rapeseed, sunflower and other oil seeds, groundnut, broad beans and leaf protein. The chapters on the third group of protein sources provide a detailed, but far from comprehensive, description of concentrates formed by biological conversion. Although domestic ruminants have not been included, because of the wealth of information already available, there are four chapters on mammals that are widely eaten, one on mammalian products that could be eaten but are not at present, and an interesting account of the uses of fungi. The final section in the volume discusses the need to provide standards, establish safety and draw up legislation to cover the processing and use of proteins. Details of regulatory bodies such as the Food and Agriculture Organization, the World Health Organization and the Protein Advisory Group, and their connexion with the *Codex Alimentarius*, are provided. The author also describes problems that have been encountered in the use of new proteins both by the industrialized nations and by developing countries. This, together with a contribution on product acceptability, provides an important and extremely relevant termination to the book.

Of particular interest to the toxicologist are those sections of the text dealing with natural toxins. The problem of the aflatoxins is outlined briefly and attention is drawn to the toxins to be found in sunflower meal and castor meal. Described in greater detail are the toxicological studies on hydrocarbon-grown yeast.

Despite its wide subject area, the book discusses the relative merits and potentialities of the various protein sources at sufficient length to allow their world role to be assessed and to provide a guide for future research. Although several protein sources have been omitted, their inclusion would probably have made the volume unmanageable. The use of the earth's protein resources has been tackled at an international level (the table of contents in English, French, Russian and Spanish reflects the approach of this International Biological Programme) and the volume provides a useful guide to both conventional and unconventional sources of food protein throughout the world.

Residue Reviews. Residues of Pesticides and Other Contaminants in the Total Environment. Vol. 56. Edited by F. A. Gunther. Springer-Verlag, Berlin, 1975. pp. ix + 138. DM 29.80.

Volume 56 of this excellent series presents a major treatise on the gas chromatography of carbamate residues, a review of the effect of sulphur dioxide on plant metabolism and structure and finally, and perhaps most notably, an account by a WHO scientist of the work and achievements of the Joint FAO/WHO Expert Committee on Pesticide Residues, with special reference to organochlorine pesticides.

Since its inception in 1961, this Joint Committee has been charged with the task of recommending acceptable daily intakes (ADIs) for man and appropriate residue limits in food for a variety of pesticidal chemicals, 114 of which had been evaluated up to and including the 1973 meeting. Brief consideration is given in this review to the conceptual meaning of the ADI, to the toxicological basis for arriving at an ADI, to the type of information required for the recommendation of residue limits in food and finally to the more recent attempts to determine actual pesticide intakes on the basis of average food consumption figures and the assumed presence of the pesticide at the recommended residue limit.

The main part of the review concludes with a presentation of toxicity profiles and ADI status for each of the following organochlorine pesticides: Aldrin, BHC, camphechlor (toxaphene), chlordane, dieldrin, endosulfan, endrin, heptachlor, lindane, chlorbenside, chlorfenson, tetrasul, bromopropylate, chlorobenzilate, chloropropylate, DDT, dicofol, methoxychlor, hexachlorobenzene, quintozene, 2,4-D and 2,4,5-T. Further reviews of other classes of pesticides already evaluated by the Joint FAO/WHO Expert Committee on Pesticide Residues are to be published in future issues of *Residue Reviews*, the next in the series being on organophosphorus pesticides.

Organochlorine Insecticides: Persistent Organic Pollutants. Edited by F. Moriarty. Academic Press Inc., (London) Ltd., London, 1975. pp. xii + 302. £7.90.

Certain parts of this wide-ranging book on organochlorine pesticides may give the impression that the ever increasing attention paid to this much maligned group of pesticides raises more questions than it could ever hope to answer. Yet anyone directly

involved in unravelling the complexities of the ecological and biological effects of the organochlorine pesticides will appreciate this paradoxical situation. Fortunately, 'persistent workers' abound in this field and we are indebted to the seven contributors to this book not only for bringing us up-to-date but also for indicating those areas requiring further exploration.

The major contribution deals with the sublethal effects of organochlorine pesticides in the thyroid. While lethal effects are widely attributed to a neurotoxic mechanism, D. J. Jefferies elaborates on his interesting hypothesis that a variety of pathological and biochemical effects seen at different sites in the body are secondary manifestations of a primary lesion in the thyroid gland. Just how hyper- and hypothyroidism and consequential hypervitaminosis A or avitaminosis A induced by some organochlorine pesticides can account for many of the seemingly unrelated effects elsewhere makes fascinating reading, but how long this hypothesis will stand the test of time is perhaps an even more fascinating question.

The other contributors concentrate on less intriguing issues—the monitoring of pesticide residues in the field, the relationship between experimental exposure of organisms and the pesticide concentrations attained in their tissues, the importance of such factors as species, strain, age and sex in determining variations in the intake, distribution, metabolism and excretion of pesticides, the effects on animal populations and, finally, an analysis of the economic considerations in the choice of pesticides and of the systems, both voluntary and statutory, employed in their control.

A. V. Holden states (p. 25) that "Chemical, and in particular analytical, science is far ahead of a full biological understanding of the effects of pollution, even though many more pollutants are probably as yet undetectable". What a daunting prospect lies ahead!

Industrial Environmental Health. The Worker and the Community. 2nd Ed. Edited by L. V. Cralley and P. R. Atkins. Academic Press Inc., (London) Ltd., London, 1975. pp. xv + 356. £13.70.

Hard on the heels of the first edition of this book, which essentially covered developments spanning 1968–1970, has come a second edition reporting on research activities and experience gained in the field of environmental and occupational health during the period 1971–1973. The word 'reporting' is used unashamedly by the reviewer because much of the ground covered in this edition is presented in the form of uncritical accounts and digests of studies published in the literature, occasionally strung together all too loosely.

Like its predecessor, the second edition embraces widely divergent topics ranging from epidemiological and toxicological studies on specific chemicals to noise, ionizing and non-ionizing radiation, personal protective devices, standards for hot working environments, the significance of domestic off-job stress and the US approach to environmental chemical control.

About one third of the book is devoted to summaries of varying depth on epidemiological studies of over 50 chemicals, including pesticides, detergents, solvents and heavy metals, and on toxicological studies in animals and man relating to a less ambitious range of chemicals, but nevertheless including many old faithfuls, such as lead, mercury, cadmium, polychlorinated biphenyls, nitrosamines and aflatoxin. But apart from these and cyclamate, saccharin, phthalate esters, ethylene oxide and HC Blue No. 1, there is little to whet the appetite of those engaged in the food, food-packaging and cosmetic industries.

Some readers might have derived greater benefit if brief introductory backgrounds and major points of interest had been included for each chemical covered and if the various discussions of analytical methodology, air sampling, toxicology, epidemiology and control measures in respect of a given chemical had been collated under that chemical rather than scattered throughout the book. The latter course tends to encourage unnecessary duplication, as has occurred in the case of carbon tetrachloride decomposition (pp. 94 & 272). Nevertheless, this book provides a useful progress report, lucidly written and well indexed, for workers concerned with the various facets of occupational and environmental health.

European Pharmacopoeia. Published under the direction of the Council of Europe (Partial Agreement). European Treaty Series No. 50. Maisonneuve S.A., Sainte-Ruffine. Vol. I (1967): pp. 401, F.fr. 130.00. Vol. II (1971): pp. xxi + 542, F.fr. 130.00. Vol. III (1975): pp. xxii + 464, F.fr. 200.00.

Volume I of the first European Pharmacopoeia was published in 1969 in accordance with the terms of the Convention on the Elaboration of a European Pharmacopoeia, under the auspices of the Council of Europe. The preface to this volume traces its background from the inception of the basic idea in 1902 through the difficult intermediate years to its final emergence as a well-produced book presenting the monographs and general methods agreed by the European Pharmacopoeia Commission up to the end of 1968.

After the list of the members of the Commission and the various groups of experts, the General Notices explain the nomenclature and abbreviations used. There is then a long and detailed section on methods of analysis, followed by a list of reagents. The actual monographs, 77 in all, commence half-way through the volume. These monographs are arranged alphabetically under their Latin names, so that while gentian root (Gentianae radix), for example, is found under G, potassium chloride must be sought under K (kalii chloridum). In each monograph, the chemical nomenclature and molecular weight, where appropriate, are followed by details of the properties of the material, of methods of identification, testing and assay and of appropriate storage conditions, a format repeated in Volumes II and III and similar to that found in the British Pharmacopoeia.

Volume II, published in 1971, includes a revised and expanded General Notices section. Both this volume and the third, published in 1975, present amendments and corrections relating to material published earlier and additional methods of analysis.

Notable additions in this connexion include, in Volume II, fluorimetry, amperometry, gas chromatography and the use of infra-red spectrophotometry and thin-layer chromatography in the identification and purity testing of steroid hormones and, in Volume III, atomic absorption spectrophotometry, electrophoresis and biological assays for corticotrophin and insulin. Volume II contains 127 new monographs, many on antibiotics, steroid hormones, vaccines and immunosera, and a long and valuable chapter on the statistical analysis of results of biological assays and tests, while its successor adds a further 111 monographs, including eight on radiopharmaceutical preparations grouped together in a separate chapter because of the special problems they present.

The order in which the various categories of monographs (e.g. chemical compounds, biological materials) appear in the Pharmacopoeia depends solely on the time at which the monographs were completed. For the chemical compounds, the nomenclature and structural formulae used follow, where possible, the rules laid down by the International Union of Pure and Applied Chemistry, and stereochemistry is shown when this affects a compound's pharmacological activity or analytical identification. Entries are indexed under various alternative headings and the indexes in the two later volumes are cumulative. The official texts are in English and French, and these three volumes and a supplement complete the first edition of the European Pharmacopoeia.

One should not underestimate the immense amount of work involved in the production of a reference manual compiled on a basis of international agreement. The result in this case may well be classed as a valuable achievement.

Mouse Hepatic Neoplasia. Proceedings of a Workshop held at the H.T.S. Management Centre, Lane End, High Wycombe (Great Britain), 12–17 May 1974. Edited by W. H. Butler and P. M. Newberne. Elsevier Scientific Publishing Company, Amsterdam, 1975. pp. xi + 195. $31.25.

The toxicological significance of the induction of tumours in the mouse by chemical agents has been a controversial topic for several years. Although the question has been raised in connexion with tumour induction in most tissues, the controversy has centred around the induction of hepatic neoplasia, primarily because of a major disagreement among pathologists about the nature of some nodular lesions induced by chemical agents. A second reason was the suspicion that some external influences caused significant fluctuations in the natural incidence of these tumours.

The book named above reproduces the proceedings of a workshop arranged to give a relatively small international group of pathologists the opportunity to identify existing areas of agreement and disagreement on the diagnosis of malignant liver tumours in the mouse and on their significance. These proceedings provide not only excellent morphological descriptions of a variety of hepatic nodular lesions but also some philosophical discussion on the meaning of malignancy in the mouse, since some malignant liver tumours seem to be well tolerated by this species.

One enlightening chapter summarizes the diagnosis made by 15 pathologists, who were given the same slides from a variety of proliferative hepatic lesions. Only five out of 43 slides were given the same diagnosis by all the pathologists! Other chapters deal at some length with the factors that affect the production of hepatic tumours. Dietary and genetic factors are by far the most important, but environmental factors can sometimes play a vital role. This point was driven home by the demonstration that one particular type of cage bedding enhanced the incidence of mouse hepatoma considerably. One puzzling feature reported was a gradual increase in the percentage incidence of hepatoma in the same outbred strain of mice over a period of 10 years, a problem worth bearing in mind when the toxicologist calls for the "background" incidence in a particular strain.

The chemical induction of hepatic tumours received extensive coverage at the meeting, their induction by nitrosamines being given particular consideration. Attention was also drawn to the possibility that cancer may develop in the mouse liver as a consequence of chronic toxic injury, and the tumours induced by two hepatotoxins, carbon tetrachloride and chloroform, were advanced as an illustration. The role of transplantability in the identification of neoplastic lesions was considered, but it seemed premature to put too much emphasis on this technique because tumours of the same histological type seemed to behave differently on transplantation.

What were the lessons learnt from this meeting? They were few but crucial. One may perhaps claim that as a result of these discussions the histological features of malignant hepatic neoplasms in the mouse are better understood, the frequency with which they metastasize is known with a bit more certainty and the ultrastructural features of some of the chemically induced tumours are better defined. The wealth of electron- and light micrographs reproduced with these lectures are of particular value in this connexion. Another important lesson concerned the value of mouse hepatic tumours as an index of carcinogenicity. In the concluding session, all the participants evaluated critically the facts and opinions proferred by the various speakers at the symposium and concluded that this particular system was not a reliable means of testing the potential hepatocarcinogenicity of chemicals of unknown activity.

This book is strongly recommended. It is essential reading for pathologists and toxicologists, particularly those engaged in making recommendations for carcinogenicity testing.

Teratology Trends and Applications. Edited by C. L. Berry and D. E Poswillo. Springer-Verlag, Berlin, 1975. pp. x 238. $35.30.

Since the thalidomide disaster at the beginning of the 1960s, there has been great concern over the possible effects of existing or novel compounds on the unborn, a situation reflected in the increasing use of teratogenicity studies in toxicology. In this monograph, an attempt has been made to review recent advances in this somewhat difficult and controversial field. The text is divided into four sections, each deal-

ing with a different aspect of teratology, although, as is perhaps inevitable in a review presenting contributions from a number of authors, there is some degree of overlap in subject matter.

The first section, entitled "New Attitudes to Experimental Teratology", begins with an introductory chapter which puts later contributions into perspective by outlining briefly certain developmental processes and the disturbance of these in relation to foetal abnormalities. The following two chapters discuss teratology in relation to genetic change, and the section is completed by an excellent account of the teratogenicity of cytotoxic agents, in which much emphasis is placed on this mechanism of action.

Problems of predictive teratology form the theme of the second section, with valuable discussions on animal model systems and on some of the difficulties encountered in the interpretation of results in relation to the human situation. The foetal environment is discussed next, with topics such as the effects of the amniotic cavity, umbilical cord and infections on normal development. This part ends with a consideration of the future prospects for foetal surgery.

The final section draws attention to some of the new aspects of developmental teratology, including late-pregnancy changes induced in the foetal central nervous system by cytotoxic agents, and enzyme polymorphism as a cause of abnormal development. The last chapter is appropriately concerned with the practical implications of the birth of an abnormal infant and offers some guidelines on the attitudes of the family and society.

This is a highly readable book which will appeal to non-specialist as well as specialist readers.

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Information Section

ARTICLES OF GENERAL INTEREST

PERSORPTION—A NEW LONG-TERM PROBLEM?

Interest in the passage of particulate matter from the intestinal lumen into the blood or lymphatics dates back to 1844, when, according to Volkheimer & Schulz (*Qualitas Pl. Mater. veg.* 1968, **17,** 17), Herbst claimed to have observed starch grains in the blood and chyle of a dog 3 hours after administration of a starch suspension. Similar observations were made later by Hirsch (*Z. exp. Path. Ther.* 1906, **3,** 390) and Verzár (*Biochem. Z.* 1911, **34,** 86), but these isolated observations were not followed up, presumably because the development of more sophisticated techniques directed attention towards the processes of diffusion and active transport in the intestinal absorption of amino acids and monosaccharides. Interest in the phenomenon has revived, however, since Volkheimer & Schulz (*loc. cit.*) administered a starch suspension orally or rectally to rats, rabbits, chickens, guinea-pigs, dogs and pigs, and examined parts of the gastro-intestinal tract histologically at various times after treatment. They found isolated starch granules lying between the cells of the epithelial layer, in the subepithelial region of the mucosa and within the lumina of the lymph and blood vessels. Furthermore, they reported a similar blood condition after self-administration of pre-cooked starchy foods such as wheat-flakes, biscuits, short-bread and crisp-bread. Of considerable interest to the toxicologist are their conclusions that the passage of particulate matter is possible wherever the intestinal mucosa is covered by a single layer of epithelial cells, that this passage may be observed primarily where the motor function of the muscularis mucosae is most pronounced and that the portal of entry is very probably in the areas of desquamation of the epithelium. The group gave this phenomenon a new name, 'persorption'.

Other observers have indicated that persorption is not limited to starch granules and that many substances of macromolecular dimensions can penetrate the epithelial layer of the intestine. Thus, Warshaw *et al.* (*Lab. Invest.* 1971, **25,** 675) used ultrastructural and histochemical techniques to demonstrate the passage of horseradish peroxidase between the epithelial cells of the small intestine of the rat, and Bockman & Winborn (*Anat. Rec.* 1966, **155,** 603) showed that ferritin particles were persorbed by the small intestine of the hamster. Passive cutaneous anaphylactic reactions were positive when egg albumin was administered orally to guinea-pigs (Bernstein & Ovary, *Int. Archs Allergy appl. Immun.* 1968, **33,** 521), indicating that some of the albumin passed through the intestinal wall, and chromatographic techniques have been used to show that insulin also can pass through the intestinal wall (Danforth & Moore, *Endocrinology* 1959, **65,** 118). Other types of particles that have been shown to pass across the seemingly intact epithelium are various grades of Sephadex (Möse *et al. Z. Immun.-Forsch.* 1972, **144,** 251), polystyrene latex particles approximately 220 nm in size (Sanders & Ashworth. *Expl Cell Res.*, 1961, **22,** 137) and polyvinyl chloride particles (Volkheimer, *Ann. N.Y. Acad. Sci.* 1975, **246,** 164).

This accumulation of evidence leaves little room for doubt that uptake of macromolecular and particulate material from the intestine does take place, and clearly the toxicologist must take this into account. Considering the vast array of protein substances in our food, it seems at first sight that persorption does not present a major toxicological problem, since relatively few types of food seem to provoke the allergic reactions that might be expected to result from the presence of foreign protein molecules in the blood. In fact, such reactions tend to be restricted to a few, particularly susceptible, individuals, and it seems that the situation pertaining to proteins may not be altogether relevant to the general question of persorption. Experiments on the absorption of bovine serum albumin and horseradish peroxidase have demonstrated that a consistent and significant decrease in uptake occurs in rats immunized with the protein in question compared with non-immunized controls (Walker *et al. Science, N.Y.* 1972, **177,** 608), suggesting that an immunological factor operates to limit the absorption of proteins and possibly to limit or prevent their systemic dissemination.

The fate of other substances is more obscure. The carrageenans, for example, appear to be persorbed by guinea-pigs, rats and monkeys but to be degraded with some difficulty by the macrophage–phagocyte system (MPS) after persorption (Grasso *et al. Fd Cosmet. Toxicol.* 1975, **13,** 195; Abraham *et al. Expl mol. Path.* 1972, **17,** 77). Furthermore, it has been shown that macrophages laden with carrageenan do not take up other macromolecular material to the same extent as unaffected cells, suggesting some impairment of the phagocytic function (Abraham *et al. loc. cit.*). These authors consider that functional impairment of macrophages may not be restricted to phagocytosis but may involve other activities in which the macrophage is known to participate (the processing of antigens for example), with unknown consequences to the organism. Although there is as yet little evidence to support these suppositions, they deserve consideration by those interested in the long-term effect of substances taken up and stored in the macrophage system, the destination of most persorbed particulate matter. Superficially at least, this problem appears to

arise also with other macromolecular additives that are known to be undegradable or are degraded only with difficulty by the mammalian digestive enzymes present in the gut or distributed systemically in the MPS. These additives include the whole range of modified starches, gums and other materials, such as alginates, used as thickeners or gelling agents. The current concern about the allergenicity of gums may well have some foundation, if the possibility that they may be persorbed and may interact with antibodies is taken into account.

Of course, not all macromolecular substances are resistant to digestion by intestinal or cellular enzymes and with those that are readily digested, storage with its possible attendant risks is unlikely to present any problem. Indeed, most foreign proteins are almost certainly degraded to component amino acids or short-chain peptides by cellular enzymes. It is important, therefore, to have an idea of the degree to which the macromolecular substances in question are amenable to enzymic degradation, if the likely fate of particles persorbed through the intestine and their consequent hazard is to be judged.

It may be argued that only a very small amount of any substance is persorbed, the implication being that such an amount is unlikely to be a hazard. By means of immunological methods, Bernstein & Ovary (*loc. cit.*) estimated that only about 1 μg of immunologically unaltered egg albumin was absorbed from a dose of 10 mg administered orally. Even taking into account the fact that a proportion of albumin is almost certainly degraded by the intestinal digestive enzymes and absorbed in a non-reactive form, the amount detected is minute. Unfortunately the other studies on proteins quoted above were predominantly qualitative and cannot be used to establish with certainty the degree of persorption, although the results support the impression that it must have been very low indeed.

However, despite the small amounts involved, the problem posed by persorption cannot be laid aside and forgotten. Most macromolecular substances in food, whether naturally occurring or added, are ingested over long periods of time, possibly for a lifetime. Under these conditions it is unwise to exclude the possibility that continued persorption of undegradable particulate or macromolecular material could result in some subtle functional disturbance of the MPS. Concern about the possible long-term toxic effects of the uptake of small amounts of various chemicals from the environment, particularly from food, has been expressed in many quarters and figured fairly recently in the report of a Joint ARC/MRC Committee on Food and Nutrition Research (*Food and Nutrition Research*, p. 167; HMSO, London, 1974). The list of such chemicals already includes compounds of lead, cadmium and mercury, and many compounds with carcinogenic properties. Perhaps it should be extended to include macromolecular substances which, if persorbed, could impair the MPS. The validity of this thinking can only be tested on the bench.

[P. Grasso—BIBRA]

VINYL CHLORIDE—PART II

The first instalment of this review (Potter, *Fd Cosmet. Toxicol.* 1976, **14**, 347) was concerned principally with the development of acro-osteolysis and haemangiosarcoma in workers exposed industrially to vinyl chloride (VC). This part continues with a description of the clinical and histological characteristics of VC-induced angiosarcoma, with animal studies on the monomer and with its probable metabolism and mechanism of action.

Angiosarcoma and its clinical and histological precursors

On the basis of a study of pathological material from a group of VC–PVC workers, Thomas & Popper (*Ann. N.Y. Acad. Sci.* 1975, **246,** 268) have reported that the livers of patients dying as a result of angiosarcoma were abnormally large, averaging over 4 kg, and were massively involved with cystic blood-filled tumours which replaced most of the liver tissue. In the larger specimens, some of the cystic spaces were several centimetres in diameter and were associated with large areas of fibrosis, haemorrhage and necrosis. The haemorrhage caused by the rupture of these cavernous cysts was the immediate anatomical cause of death in several patients.

Prior to tumour development, the histological alterations of the liver following VC exposure are in most cases relatively discrete and unspecific (Gedigk *et al. ibid* 1975, **246,** 278). Degenerative lesions, adaptive responses and fibrosis occur, as in many other types of hepatotoxic damage, but the degenerative areas are sharply demarcated from the undamaged parenchyma; this pattern of degenerative lesion in conjunction with the activation and proliferation of the sinusoidal cells and hepatocytes may be considered characteristic of VC damage. The proliferation of sinusoidal cells is similar to that seen in arsenic poisoning.

The severity of the degenerative lesions, development of septal fibrosis and changes in the sinusoidal cells have all been found to be dependent on the time of exposure to VC. However, in contrast to the degenerative changes, which were found to be reversible upon removal of the patient from VC exposure, the proliferative activity does not appear to decrease and may finally converge into a tumour. From studies of the livers of workers exposed to VC, it has been suggested that the fibrosis may be a precursor in the development of angiosarcoma (Popper & Thomas, *ibid* 1975, **246,** 172), a probability supported by findings in some animal studies (see below). The persistence of proliferative activity was thought to occur only after severe exposure of sufficiently long (>10 years) duration (Gedigk *et al. loc. cit.*). In a single

case of lesser exposure (lasting 5·5 years) to high levels of VC, the activation of both hepatocytes and sinusoidal lining cells was virtually absent after cessation of exposure, although fibrosis persisted (Berk *et al. ibid* 1975, **246**, 70).

Histological features associated specifically with angiosarcoma have suggested a continuous spectrum of changes initially manifest by multifocal areas of stimulated sinusoidal cells followed by increasing degrees of sinusoidal cell atypia and proliferation associated with sinusoidal dilatation and culminating in a progressively growing infiltrative angiosarcoma with a sinusoidal growth pattern. Further growth of the sinusoidal type of angiosarcoma results in the observed papillary and cavernous growth patterns (Thomas & Popper, *loc. cit.*). The series of changes in the liver appear to be multicentric, but only some of the lesions progress to fully developed angiosarcomas that become clinically evident. Because of the multicentricity of this development, however, surgical treatment is not possible and chemotherapy must be relied upon. In one patient deposits of angiosarcoma were observed in many other organs in addition to the liver and results of further investigations of this nature are awaited (Thomas & Popper, *loc. cit.*). These authors consider that at least some of these extrahepatic tumours were independent primary neoplasms, but this view has not yet been confirmed.

The pathway by which VC activates and stimulates the sinusoidal cells and the hepatocytes, and the correlation between the two, are still unknown (Gedigk, *loc. cit.*; Thomas & Popper, *loc. cit.*) but animal studies are increasing our understanding of the mechanisms involved.

Animal studies

The pioneer study on exposure of rats to VC (*Cited in F.C.T.* 1972, **10**, 601) involved exposure to high concentrations (30,000 ppm) for 4 hours daily on 5 days/week over a 12-month period. Subsequent interpretation (Maltoni & Lefemine, *Ann. N.Y. Acad. Sci.* 1975, **246**, 195) suggested that the malignant skin tumours observed in this study were carcinomas arising from the Zymbal gland (a sebaceous gland of the exterior acoustic duct), which is responsive to a large number of carcinogens, and that the malignancies of the lung were metastases from Zymbal-gland tumours.

Preliminary results of experiments designed to study the effects of VC administered to animals by different routes and under different conditions have now been reported (Maltoni & Lefemine, *loc. cit.*). VC administered by inhalation produced a range of tumours in all the animal species studied (rats, mice and hamsters), liver angiosarcomas being observed in all three species. Repeated daily exposure comparable to that used in the early test had a carcinogenic effect in both rats and mice at levels down to 50 ppm, the incidence of angiosarcomas and nephroblastomas being dose related in the lower part of the dose range. Initial results in the rat also showed that the neoplastic response was affected by the length of exposure to VC, that it varied with different strains and that there was apparently a transplacental effect. During the discussion arising from the presentation of his paper at a symposium on VC (*Annals of the New York Academy of Sciences* 1975, **246**, 221), Professor Maltoni explained that fibrosis was observed only in some of the livers in which angiosarcomas were present and was thought to be a transitional stage in tumour development following exposure to the lower levels of VC. Administration of VC by oral intubation (*Food Chemical News* 1975, **16**(52), 46) also resulted in the occurrence of angiosarcoma.

The effects of a single 6-hour exposure to 50,000 ppm VC in air on rats pretreated with phenobarbitone (PB) have been studied (Jaeger *et al. Nature, Lond.* 1974, **252**, 724). Acute biochemical and histological changes were observed in these rats, but in rats that had not been pretreated, exposure to the same level of VC caused no abnormality. In PB-treated rats, marked vacuolization of the centrilobular parenchymal cells and focal necrosis of the mid-zonal parenchyma were observed. PB induces components of the hepatic mixed-function oxidase system, an intracellular enzyme complex located within the membranes of the endoplasmic reticulum. The observation that PB enhanced injury from VC exposure, coupled with the fact that acute VC-induced liver lesions involve the endoplasmic reticulum, suggests that this organelle is the primary site of generation of toxic metabolites from VC, as it is known to be for other halogenated hydrocarbons. Ultrastructural observations (Reynolds *et al. Envir. Hlth Perspect.* 1975, **11**, 227) have supported this hypothesis.

Rate pretreated with PB and exposed to VC at a level of 50,000 ppm for 6 hours on five consecutive days showed no biochemical abnormalities, and histological findings were not indicative of recurrent acute injury, suggesting that injury following the first exposure to VC protects against further injury for at least 5 days and allows healing of the initial lesion (Jaeger *et al. loc. cit.*).

Similar experiments were carried out involving pretreatment either with PB or with 3-methylcholanthrene, another inducer of the mixed-function oxidase system (Drew *et al. Envir. Hlth Perspect.* 1975, **11**, 235). After exposure to 13,500 ppm VC for 6 hours/day for 10 days, morphological changes in the liver similar to those resulting from a single exposure, as described by Jaeger *et al.* (*loc. cit.*), were observed in only two rats, both from the group of four pretreated with PB. Of the other parameters investigated, including growth rate, organ weights and biochemical changes, only growth rates showed a significant difference from controls. On day 3 of the VC exposure, a marked decrease in growth rate was observed in PB-pretreated rats, but not in the other groups.

Metabolism and mechanism of action

Data on the structure–activity relationships of direct-acting alkylating carcinogens and on the mechanism of action of chemical carcinogens in general have been reviewed for their relevance to VC (Van Duuren, *Ann. N.Y. Acad. Sci.* 1975, **246**, 258). Since VC is a small relatively unreactive molecule compared with known direct-acting carcinogens, it seems likely that metabolism to activated carcinogenic intermediates occurs. Likely intermediates, particularly in the liver, are epoxides, since although epoxidizing enzymes are present in many organs and tissues at low levels, they are particularly rich in the

liver. A favoured structure for the active carcinogenic intermediate is chloroethylene oxide: $H_2C(O)CHCl$. Since this is not only an epoxide but also an α-haloether, it belongs to two groups of compounds known to include carcinogens. A similar compound has been suggested as a major intermediate of trichloroethylene.

Like other α-haloethers, chloroethylene oxide has a strong alkylating potential and a very short half-life (1·6 minutes) in neutral aqueous solution, comparable with the half-life of less than 2 minutes for bis(chloromethyl) ether (Huberman *et al. Int. J. Cancer* 1975, **16,** 639).

Preliminary studies (Hefner *et al. Ann. N.Y. Acad. Sci.* 1975, **246,** 135) on the fate of VC inhaled by rats indicated that, when present at levels below 100 ppm in the inhaled air, it was metabolized mainly via the alcohol dehydrogenase pathway, involving sequential oxidation to 2-chloroethanol, chloroacetaldehyde and monochloroacetic acid. At higher exposure levels, this pathway appeared to be saturated and it was speculated that one alternative pathway might involve the direct epoxidation of VC by microsomal oxidases, and subsequent rearrangement of the resulting chloroethylene oxide to chloroacetaldehyde.

Isolated chloroethylene oxide rearranges spontaneously to chloroacetaldehyde, which will react with glutathione to give *S*-carboxymethylglutathione as a final metabolic product (Van Duuren, *loc. cit.*). This is consistent with the reduction of non-protein sulphydryl levels in the livers of VC-exposed rats, and with the detection of metabolites conjugated with glutathione and/or cysteine in the urine (Hefner *et al. loc. cit.*).

If the inferences of Hefner *et al.* (*loc. cit.*) are correct, the metabolism of VC to carcinogenic intermediates only after saturation of a primary metabolic pathway, which produces no carcinogens, offers hope for the existence of a no-effect level for VC.

Since angiosarcoma of the liver is a rare disease in man, consideration of other chemicals that cause the same type of tumour is worthwhile. Arsenic causes liver effects similar to those of VC and the mechanism for its toxicity has been shown to be via its reaction with 6,8-dithio-octanoic acid (*a*-lipoic acid), in which arsenic forms a stable bridge between two sulphydryl groups. Chloroethylene oxide would react similarly with α-lipoic acid (Hefner *et al. loc. cit.*).

It is commonly accepted that many chemical carcinogens exert their effects by binding covalently to cellular macromolecules. Support for this in connexion with VC intermediates has been obtained by incubation of ^{14}C-labelled VC with rat-liver microsomes (Bolt *et al. Lancet* 1975, **I,** 1425). VC metabolites were observed to bind covalently to microsomal protein, and to other SH-containing proteins and also to RNA if these were added, but only in the presence of NADPH. Such covalent binding to protein was depressed by the addition of glutathione and by an inhibitor of the microsomal mixed-function oxidizing system (Kappus *et al. Nature, Lond.* 1975, **257,** 134).

O_2^- radicals are known to be involved in epoxidation by the microsomal enzyme system and appear to convert VC to a metabolite which binds covalently to a protein like albumin (Kappus *et al. loc. cit.*). Experiments using the xanthine-oxidase model system, which generates H_2O_2 and the O_2^- radical, bound VC metabolites to albumin, whereas a control experiment with albumin and H_2O_2 did not, thus offering further support for the hypothesis of an epoxide intermediate.

Mutagenicity

Most chemical carcinogens show a mutagenic effect after metabolic activation (de Serres, *Mutation Res.* 1975, **33,** 11). *In vitro* mutagenicity studies in strains of *Salmonella typhimurium* on VC incubated with the 9000 g supernatant of liver homogenates from rat, mouse and man indicated (Bartsch *et al. Int. J. Cancer* 1975, **15,** 429) that the liver will efficiently convert VC into mutagenic metabolites. It is not clear, however, which VC metabolites are responsible, nor whether these are identical with those responsible for carcinogenicity. The results with livers of PB-pretreated animals again suggest that microsomal mixed-function oxidases play a role in VC biotransformation.

Mutagenicity studies with specific metabolites of VC showed chloroethylene oxide to cause a mutagenic response with a much lower toxicity than was exerted by an equimolar concentration of chloroacetaldehyde, thus demonstrating that chloroethylene oxide acts as a mutagen *per se* and not exclusively via its spontaneous rearrangement product, chloroacetaldehyde. Both of these compounds were strongly mutagenic compared with 2-chloroethanol and monochloroacetic acid (Huberman *et al. loc. cit.*).

A study of 56 workers exposed to various levels of VC and compared with 24 not so exposed (Purchase *et al. Lancet* 1975, **II,** 410) suggested that VC might have a detectable effect on chromosomal aberrations in man. Lymphocyte cultures prepared from blood samples from both groups showed a significantly ($P < 0{\cdot}05$) increased percentage of B, Cu and Cs cells in the exposed workers.

In a dominant lethal study in mice, males exposed to 3000–30,000 ppm VC for 6 hours/day on five consecutive days were mated with non-exposed females (Purchase *et al. loc. cit.*). There was no significant increase in the number of early deaths per implantation compared with a control group not exposed to VC. Thus the mutagenic effects of VC appear not to occur in the germ cells, possibly because active metabolites responsible for the toxic effects do not reach the testis. A potential danger to the foetus from mutagenic effects transmitted via the sperm does not seem, therefore, to exist.

Exposure levels

Analytical methods for the detection of VC in beverages, vegetable oils and vinegars have been developed using gas–liquid chromatography with a flame-ionization detector, confirmation being possible by mass spectrometry (Williams & Miles, *J. Ass. off. analyt. Chem.* 1975, **58,** 272). VC was only detected in foodstuffs packaged in such a way that there was contact with PVC. Levels in alcoholic beverages ranged from 0 to 1·6 μg/ml, in vinegar from 0 to 8·4 μg/ml and in peanut oil from 0·3 to 3·3 μg/ml. It

was not possible to assess which foodstuffs were more susceptible to VC contamination, owing to the large variety of types of PVC used.

Controlled storage tests using PVC containers of known residual monomer content (Tester & Moffitt, Paper 4b, presented to the BPF/PRI Joint Conference on "Vinyl Chloride and Safety at Work", held at Hayes, Middx, on 28 May 1975) have shown that levels of extraction of VC into the contents are of the same order for a wide range of foodstuffs and that the rate of extraction increases with temperature. Maximum extraction levels after long periods of time indicate a partition between the PVC container and its contents, such that the concentration of VC is always much higher in the PVC. At 23°C, foodstuffs stored in containers made of PVC with a residual monomer content of 30 ppm gave maximum concentrations of VC in the contents as follows: water, 0·14 ppm; orange squash, 0·05 ppm; maize oil, 0·10 ppm. Margarine stored in containers with residual VC contents of about 80 ppm contained a maximum of 0·08 ppm VC at the container wall (0·05 ppm in the centre of the margarine) when stored at room temperature and 0·01 and 0·006 ppm, respectively, when stored at 5°C. Residual monomer concentrations in PVC bottles of current manufacture are 5 ppm or less and in PVC containers for margarine 10 ppm or less.

A higher exposure to VC is likely to occur through the use of aerosol sprays containing VC. Analysis of air concentrations (Gay *et al. Ann. N.Y. Acad. Sci.* 1975, **246**, 286) showed that the user of such sprays is exposed to high peak concentrations of VC even with good room ventilation, and in smaller rooms the concentration persists for some time. A decrease in VC concentration with time appeared to be a dilution effect of room ventilation. The peak concentration recorded within the breathing zone was 380 ppm 1 minute after a 30-second release of spray.

Legislation and recommendations on VC

Current uncertainties about the effects of exposure to low levels of VC are reflected in recommended maximum working concentrations. Within the EEC countries, the maximum allowable concentration (MAC) for VC varies between 5 and 100 ppm (*Official Journal of the European Communities* 1975, **18**, C192/35). In the UK, the levels have recently been revised to a 30 ppm ceiling value and a 10 ppm time-weighted average (Vinyl Chloride: Code of Practice for Health Precautions, as amended. Health and Safety Executive, London, 1975). The USA has adopted a lower (threshold limit) value of 1 ppm (*Federal Register* 1974, **39**, 35889), the level also selected by Sweden (*Official Journal of the European Communities* 1975, **18**, C192/35). In comparing such differences in levels, however, stringency of monitoring procedures must be considered. The UK code of practice (*loc. cit.*) states that efforts should be made to reduce VC exposure levels to zero, that regular monitoring data should be made available to employees, and that data on workers exposed to higher levels of VC, all of whom should wear protective clothing, should be kept for at least 30 years.

Data on migration of VC from different types of PVC container to specific foodstuffs is lacking and hence regulations or recommendations are scarce. In Canada, it is proposed to prohibit the sale of food in packages that may yield any amount of VC to the food (Health Protection Branch, Department of Health and Welfare, Information Letter no. 439, 1975). In the USA, testing of PVC products is in progress to comply with proposed restrictions, which will limit the use of PVC in contact with food to cases where potential migration of VC from container to food is negligible (*Food Chemical News* 1975, **17**(31), 15). Such regulations, of course, depend upon the limits of detection of analytical techniques, which may be expected to decrease as methods improve.

Conclusion

In the 3-year period following the first reports of liver angiosarcoma in PVC-production workers, concern over VC toxicity has rapidly increased. The results of experimental and epidemiological studies are of necessity preliminary, but certain points have been established. The association of angiosarcoma incidence with VC exposure has been substantiated by the results of animal experiments, and available data point to the implication of chloroethylene oxide as a carcinogenic intermediate. Further results of such studies are awaited, but of greater urgency is the development of a test for the early diagnosis of angiosarcoma and of its precursor lesions in persons exposed to VC.

Industrial exposure to VC has, in general, been greatly reduced, but ultimate legislation will depend on whether a no-effect level for VC can be established. The major source of domestic exposure is VC-containing sprays. VC is also present in some foodstuffs, as a result of contact with PVC, but forthcoming legislation is likely to reduce the permitted levels to below analytical limits of detection.

[H. R. Potter—BIBRA]

MORE ON PHTHALATE ESTERS

Phthalate esters are commonly included as plasticizers in PVC used for medical and surgical equipment. It has been shown that di-2-ethylhexyl phthalate (DEHP), a common plasticizer in PVC film, can migrate into blood stored in PVC transfusion packs (*Cited in F.C.T.* 1971, **9**, 910; *ibid* 1973, **11**, 914), and that it has a significant retention time in tissues (*ibid* 1975, **13**, 146), particularly in those of critically sick infants (*ibid* 1976, **14**, 71).

Leaching problems

The Lancet (1975, **I**, 1172), commenting that plasticizers may constitute up to 40% of the weight of PVC formulations and may be leached from PVC tubing

in contact with body tissues, points out that DEHP passes into certain blood fractions and into iv administered fluids in general, and its ability to modify or abolish vascular response to physiological stimuli may induce ischaemia and tissue necrosis in sick infants. The *British Medical Journal* (1975, **3**, 262) suggests that storage bags made of PVC may present a toxic hazard during transfusion, extracorporeal circulation or umbilical catheterization of neonates. Gilbo & Coles (*Vox Sang.* 1975, **29**, 242) found measurable amounts of DEHP in stored blood plasma before the normal 21-day expiry date had been reached. Most of the leached DEHP appeared in Cohn fractions III and IV, in which plasma lipoproteins concentrate. These two fractions are usually discarded in favour of fractions that are therapeutically more useful, thus affording some protection against DEHP toxicity. In an examination of haemodialysis tubing made of PVC plasticized with diethyl phthalate (DEP), Neergaard *et al.* (*Nephron* 1975, **14**, 263) found that most of the DEP released appeared in the perfusate within 1 hour. In consecutive perfusion experiments using a standard volume of sterile water the DEP release became negligible after five perfusions each of 1 hour, but when the period was increased to 24–96 hours, almost constant concentrations of DEP were present in the perfusate during five successive perfusions. Two to four periods of perfusion within 24 hours, simulating more closely the dialysis procedure, resulted in almost constant DEP release from the tubing. After long perfusion times a maximum concentration of about 50 mg DEP/litre of perfusate was demonstrated.

Distribution, retention and excretion

Williams & Blanchfield (*J. agric. Fd Chem.* 1975, **23**, 854) gave ^{14}C-labelled dibutyl phthalate (DBP) to rats as a single oral dose of 0·1–0·13 g/kg and observed a general body distribution of radioactivity followed by rapid clearance of 80–90% in the urine within 48 hours. Urinary metabolites detected were phthalic acid and monobutyl, mono-3-hydroxybutyl and mono-4-hydroxybutyl phthalates. There was no evidence of accumulation of DBP or monobutyl phthalate in the tissues or organs of rats fed 0·1% DBP in the diet for 12 weeks. Tanaka *et al.* (*Toxicology* 1975, **4**, 253) reported that 80% of a single oral or iv dose of [^{14}C]DEHP given to rats was excreted in the urine and faeces within 5–7 days. In rats whose bile duct was cannulated, 5 and 24% of the dose appeared in the bile within 24 hours of oral and iv dosage, respectively. There was no detectable excretion in lung exhalations. Intravenous administration was followed by transitory localization of 70–80% of the activity in the liver after 1 hour, the level declining rapidly thereafter. There was also transitory accumulation in the intestine, which initially increased in radioactivity as the liver activity decreased. Clearance of activity from adipose tissue was relatively slow. The lowest ^{14}C concentrations appeared in the testes and brain. After oral dosage, faecal excretion was of almost completely unchanged DEHP, but four major metabolites were distinguished in urine. No significant accumulation was observed in the tissues or organs. In mallard ducks fed 10 ppm DBP or DEHP for 5 months a random analysis of tissues revealed no significant DBP or DEHP in heart muscle, lung, subcutaneous fat or breast muscle (Belisle *et al. Bull. env. contam. & Toxicol.* (*U.S.*) 1975, **13**, 129). It can be assumed, therefore, that neither DBP nor DEHP accumulates appreciably in mallard tissues.

Transplacental transfer of DEP and DEHP has been demonstrated by Singh *et al.* (*J. pharm. Sci.* 1975, **64**, 1347) in rats given an ip injection of the ^{14}C-labelled phthalates on day 5 or 10 of gestation. Examination of mothers and foetuses from days 8 and 11, respectively, showed the presence of the phthalates and their metabolites in maternal blood, placenta, amniotic fluid and foetus at all stages of gestation. Excretion of phthalates from these tissues was in accordance with a first-order elimination curve from which a half-life for DEP of 2·22 days and for DEHP of 2·33 days was calculated.

Toxicity studies

In an extensive toxicity study, Lawrence *et al.* (*Envir. Res.* 1975, **9**, 1) reported acute ip LD_{50} values in mice for dimethyl phthalate (DMP), DEP, DBP and di-isobutyl phthalates (3·22–3·99 g/kg), for di-2-methoxyethyl and ethylcarboxymethyl phthalates (4·18 and 4·38 g/kg), for butylcarbobutoxymethyl phthalate (BCBP; 6·88 g/kg), for DEHP (37·77 g/kg) and for di-*n*-octyl phthalate (DOP; 65·70 g/kg). Dinonyl, di-isodecyl and diundecyl phthalates were insufficiently toxic at 100 g/kg to permit the calculation of LD_{50} values. Of the phthalates studied, intradermal injections of only DMP, DEP and di-3-methoxyethyl phthalate produced significant irritation in mice, and none irritated the rabbit eye. No significant irritation appeared at the ip injection sites of these phthalates, but DEHP and DOP showed some evidence of mild transient serosal irritation after 7 days. Chronic toxicities, determined after daily ip injection, were two to four times the acute toxicities for most phthalates, but increased dramatically for DOP and DEHP to 22 and 28 times the acute toxicity, respectively. Pretreatment of mice with ip injections of phthalates had variable effects on phenobarbitone sleeping time; di-isobutyl phthalate proved unique in reducing sleeping time, possibly by enzyme induction, while other phthalates increased it. No abnormalities were seen in the lungs of mice after inhalation of phthalates.

Growth of human diploid fibroblast cultures has been significantly but reversibly inhibited by a 0·15-mM concentration of DEHP, a level that may be attained in blood stored in PVC packs for 21 days at 4°C (Jacobson *et al. Res. Commun. chem. Path. Pharmac.* 1974, **9**, 315). Active division was resumed when fibroblasts that had been incubated with 1·56 mM-DEHP for 6 days were washed and re-incubated. Platelet concentrates maintained at 22°C in PVC packs contained up to 0·70 mM-DEHP after 48 hours. More cell-culture experiments have been described by Jones *et al.* (*Toxic. appl. Pharmac.* 1975, **31**, 283), who tested the effect of DEHP and BCBP on the human diploid cell strain WI-38. At 70 μM-DEHP and at 12 μM-BCBP, growth was reduced by 50%. The toxicity of these phthalates was greater in a replicating cell population than in a non-replicating confluent cell layer. WI-38 cells transferred to control medium after incubation with 160 μM-DEHP for 3

days showed a growth rate that was only 60% of the normal after 5 days in the DEHP-free medium, but the subsequent growth of cells previously incubated with 14 μM-BCBP for 3–5 days was not reduced.

Cultures of rat cerebellar tissue showed depression of the network formation of nerve fibres, with normal outgrowths of glial cells and fibroblasts, after addition of 0·31 mM-DMP to the medium (Kasuya, *Bull. env. contam. & Toxicol.* (*U.S.*) 1974, **12,** 167). At 0·61 mM-DMP, network formation was further depressed, and at 1·83 mM- and 3·05 mM-DMP this depression was often accompanied by incomplete inhibition of glial outgrowths. DEP at 0·26 mM depressed fibroblast sheet development without affecting the outgrowth of glial cells and nerve fibres, while at 0·51 mM nerve fibres sometimes did not develop and fibroblasts did not form sheets. At 1·53 mM and 2·55 mM, DEP strongly depressed nerve-fibre growth but did not induce degenerative changes. DBP at 0·20 mM produced no change in nerve fibres, glial cells or fibroblasts, but at 0·39 mM, and even more at 0·78 mM, granulation of fibres and depression of outgrowths of fibroblasts appeared. On this basis, therefore, DBP was the most and DMP the least toxic of these three esters.

Environmental considerations

Aquatic invertebrates are essential for the maintenance of the population of higher animals, and the effect of phthalates in the environment may be to reduce their growth and reproduction. Sugawara (*Toxic. appl. Pharmac.* 1974, **30,** 87) has reported the effects of incubating the eggs of the brine shrimp (*Artemia salina*) at 26°C for 40 or 72 hours with 2% sodium chloride containing 10–50 ppm of various phthalates. Neither DMP at 50 ppm nor DEP at 20 ppm affected egg hatching, whereas DEP at 50 ppm significantly reduced hatching rate. DBP inhibited hatching at all concentrations and was the most toxic of the phthalates tested. Phthalate toxicity could not be correlated with the water solubility of the esters. In a further paper, Sugawara (*Bull. env. contam. & Toxicol.* (*U.S.*) 1974, **12,** 421) confirmed the descending order of toxicity for the eggs of the brine shrimp as DBP, DEP, DMP, thus supporting the relative toxicities determined in fibroblast culture by Kasuya (*loc. cit.*), and found that this order applied also to the nauplius (larva) of the shrimp.

In the environment the possible metabolism of phthalate esters by micro-organisms is of importance in determining residual toxicity. Engelhardt *et al.* (*Bull. env. contam. & Toxicol.* (*U.S.*) 1975, **13,** 342) reported that incubation of DBP with *Penicillium lilacinum*, *Corynebacterium petrophilum* ATCC 19080, *Arthrobacter hydrocarboglutamicus* ATCC 15583, *Mycobacterium phlei* and other coryneform bacteria produced the metabolite mono-*n*-butyl phthalate, which was degraded further by various species of Pseudomonas and Brevibacterium and some coryneforms to protocatechuic acid (3,4-dihydroxybenzoic acid). The degradation of phthalates by mixed bacterial populations in soil and water therefore seems to be relatively easy when compared with that of many environmental contaminants, such as the persistent organochlorine compounds.

[P. Cooper—BIBRA]

BACK TO DIMETHYLSULPHOXIDE

Dimethylsulphoxide (DMSO) offered great promise some years ago as a vehicle for percutaneous administration of drugs. It fell into disrepute, however, and clinical trials came to a halt when experimental evidence revealed that DMSO caused damage to the lens of the eye in dogs, rabbits, pigs and hamsters, but not monkeys, after repeated administration (*Cited in F.C.T.* 1968, **6,** 790). DMSO administered by mouth or by sc injection is rapidly absorbed and is eliminated as free DMSO and as the metabolites, dimethyl sulphide (DMS) and dimethyl sulphone ($DMSO_2$) (*ibid* 1968, **6,** 791). DMSO has also been shown to increase water loss through the skin (*ibid* 1968, 6, 679) and to cause dermal vasodilatation with increased permeability and variable degrees of oedema (*ibid* 1972, **10,** 735). More recent studies have been designed to establish the limits of DMSO toxicity, with a view to the possible reinstatement of this solvent as a therapeutic and experimental agent.

Metabolism and excretion in cattle

In dairy calves and cows receiving topical, sc or intramammary doses of ^{14}C-labelled DMSO, elimination followed a similar pattern for each route of administration, but this pattern was dissimilar to that in other species (Tiews *et al. Ann. N.Y. Acad. Sci.* 1975, **243,** 139). Elimination was more rapid after sc or intramammary injection than after topical application, and was almost entirely via the exhaled gases and urine, a negligible proportion being found in the faeces and a little (2%) in milk. In a calf slaughtered 20 days after application of [^{14}C]DMSO, the only tissues in which detectable levels of radioactivity persisted were the subcutaneous- and bowel-fat deposits. The excretion of DMSO and its metabolites in the urine reached a peak within 6–12 hours, decreased sharply thereafter and was complete within 12–28 hours. Excretion of ^{14}C activity in the exhaled air followed a similar pattern, the radioactive compound being presumed, from its odour, to be DMS, while excretion of $DMSO_2$ in the urine reached a peak after 30–40 hours in calves and 6–12 hours in cows, returning to physiological levels within 7 and 3–5 days, respectively.

Effects on hepatic disposition of carcinogens

The use of DMSO as a solvent for polycyclic hydrocarbon carcinogens raises the question of its effect on the disposition of these compounds. Experiments described by Levine (*ibid* 1975, **243,** 185) indicate that DMSO is effective in altering the binding of carcinogens to liver fractions only when the carcinogen

is dissolved in DMSO for injection. In female rats, biliary excretion of labelled 3-methylcholanthrene (MC) was more rapid after iv injection of MC dissolved in DMSO than after injection of a 1% suspension in albumin, but injection of DMSO alone had no such effect on the biliary excretion of MC injected subsequently. The livers of rats injected with 2·5 mg MC/kg showed nearly twice as much activity bound to the microsomal fraction after 5 minutes when DMSO was used as solvent as when an albumin suspension was used. Enhanced microsomal binding of MC was also apparent in a liver homogenate to which a DMSO solution of the carcinogen was added directly. A parallel effect was noted with labelled benzo[*a*]pyrene. Thin-layer chromatography of DMSO solutions of the carcinogens indicated that a DMSO–carcinogen complex was formed with microsomal-binding properties differing from those of the carcinogen itself. These findings prompt caution in the experimental use of DMSO as a solvent for otherwise intractable compounds.

Animal toxicity studies

Further investigations of DMSO toxicity in dogs, pigs, rats and rabbits have been reported by Noel *et al.* (*Toxicology* 1975, **3**, 143). Oral intubation with 50% aqueous DMSO at a level of 9·0, 3·0 or 1·0 ml DMSO/kg/day on 5 days/week was carried out in dogs for up to 2 years and in rats for up to 18 months. Dermal applications of 50 or 90% aqueous DMSO were made daily to rabbits for 6 months and two equal doses were applied daily on 5 days/week to pigs for 12 months at total daily dose levels of 8·1, 4·5, 2·7 or 1·5 ml DMSO/kg.

In dogs there was one accidental and fatal lung aspiration of DMSO. Occasional vomiting occurred at 9 ml/kg/day and transitory head-shaking was seen during weeks 11 and 12 at this and at the 3 ml/kg/day level. There were no adverse effects on body weight or food consumption. The two highest doses provoked persistent diuresis, which may explain a finding of increased red cell volume and haemoglobin at the 9 ml/kg/day level. With these two doses, bilaterally symmetrical changes in the eye lens appeared during weeks 5–10, commencing with refractive changes and progressing to involve the adjacent cortex and to cause opalescence of the lens nucleus. After 9–10 months, changes in the vitreous humour appeared as linear cracks behind the lens. The transient appearance of peripheral (equatorial) lenticular cataract in dogs after 5 months on the highest dose level could not be explained.

The weight gain of pigs on the highest dermal dose of DMSO was depressed for the first 18 weeks but not afterwards. There was no evidence in pigs of lens opacities, opalescence or vitreous changes. Slight changes in refraction appeared after 13 weeks on the highest dose and became severe after 12 months. Lower doses caused mild to moderate changes in refraction. In rats, the only ophthalmic effect observed was a mild alteration in lens refraction in the high-dosage group. Toxic effects in rabbits were confined to the lens, which developed refractive changes. The incidence of these was not affected by abrasion of the treated area of skin.

Noel *et al.* (*loc. cit.*) concluded their report of these animal tests by discussing the implications of these results for future studies on the reversibility of effects and safety assessment of compounds toxic to the eye.

Human toxicology

Brobyn (*Ann. N.Y. Acad. Sci.* 1975, **243**, 497) has described a study in 65 healthy volunteers who received a dermal application of an 80% DMSO gel providing 1 g DMSO/kg/day for 14 days. This was estimated to represent 3–30 times the dose used in human treatment. Blood and urine studies after 7 and 14 days indicated no toxic changes. Ten subjects receiving DMSO, but no controls, showed eosinophilia, which was attributed to the cutaneous histamine-releasing effect of DMSO. Ten test subjects and one control showed an occasional large lymphocyte with deeply basophilic cytoplasm, concluded to be the result of a minor viral infection and unrelated to DMSO. There were no significant changes in the results of slit-lamp and ophthalmoscopic examination, in visual field, in refraction or in intraocular tension. Vague conjunctival irritation, of which some subjects complained, was attributed to inadvertent transfer of DMSO gel from the fingers after application to the skin. There was a variable degree of skin reaction, involving whealing and erythema, drying and scaling, followed by a return to normal skin appearance within 3 weeks. A few subjects showed a mild reduction in systolic blood pressure, attributed to increased cutaneous circulation. Some sedation, headache, nausea and dizziness were reported by the participants.

Forty subjects then completed a separate 90-day study, in which 80% DMSO was again applied to the skin at 1 g DMSO/kg/day. No significant abnormalities appeared, apart from eosinophilia in 51% of the test subjects and 31% of the controls. An occasional large lymphocyte was detected in 44 and 42%, respectively, so that DMSO was unlikely to have contributed to this phenomenon. On the strength of these findings, Brobyn (*loc. cit.*) asserts that DMSO can be pronounced safe for man, and that the next necessity is a demonstration of its efficacy as a therapeutic agent for specific indications.

[P. Cooper—BIBRA]

Fd Cosmet. Toxicol. Vol. 14, pp. 505–517. Pergamon Press 1976. Printed in Great Britain

TOXICOLOGY: BIBRA ABSTRACTS AND COMMENTS

PRESERVATIVES

3053. Tannins enhance nitrosamine formation

Walker, E. A., Pignatelli, B. & Castegnaro, M. (1975). Effects of gallic acid on nitrosamine formation. *Nature, Lond.* **258,** 176.

Tannins were earlier shown to inhibit nitrosamine formation *in vitro* (Bogovski *et al.* in N-*Nitroso Compounds Analysis and Formation*, edited by Bogovski, Preussman & Walker; IARC Scient. Publ. 1972, no. 3, p. 127). In that study, the incubation of dimethyl- or diethylamine and nitrite at pH 2·4–4·3 for 10 days with a 0·5% solution of tannin, or with tea, beer or apple juice containing about 0·013–0·15% natural tannins, resulted in a far lower nitrosamine yield than did incubation with water alone. On the other hand, the naturally occurring polyphenol, chlorogenic acid, in 0·01 M concentration had a powerful catalytic effect on the *in vitro* formation of *N*-nitrosopiperidine at pH 4 after only 10 min (Challis & Bartlett, *Nature, Lond.* 1975, **254,** 532).

In the study cited above, nitrite (0·075 M) and diethylamine (0·5 M) were incubated in buffered aqueous solutions of pH 2–7, alone or with the addition of the polyphenol, gallic acid (GA), a naturally occurring gallotannin. The high amine concentration allowed the use of a low nitrite concentration, so that the competitive reaction of nitrite with GA was not masked. After 30 min, the solution was made alkaline to stop further reaction and extracted with methylene chloride. Determination of the nitrosamine concentration of the extract by UV spectrophotometry and gas chromatography revealed that GA had a strongly catalytic effect on nitrosamine formation which was, however, restricted to a narrow pH range in the region of pH 4. Moreover, the yield of nitrosamine decreased almost linearly with increasing concentrations of GA, although at 0·0625 M-GA it was still double that in water alone, thus demonstrating the balance between the retarding and catalytic effect of the polyphenol. Similar results were obtained when tannic acid was used in place of GA. The effects of dietary tannins on nitrosamine formation *in vivo* would thus be expected to vary greatly, depending on the pH and on the concentrations of tannins, nitrite and nitrosatable amines ingested simultaneously or formed in the body.

MISCELLANEOUS DIRECT ADDITIVES

3054. No relation between hyperkinesis and food additives

Palmer, S., Rapoport, J. L. & Quinn, P. O. (1975). Food additives and hyperactivity. *Clin. Pediat.* **14,** 956.

It has been reported that the FDA has withdrawn from participation in a planned study of the alleged association between food-additive consumption and hyperkinesis and learning difficulties in children (*Food Chemical News* 1974, **16**(39), 30). The preliminary conclusions of the US Advisory Committee on the Relationship of Diet to Hyperkinesis were that such a relationship has not been demonstrated hitherto by controlled studies, that a diet free of salicylates and food additives does not improve hyperkinetic children and that the dietary measures recommended by Dr. B. F. Feingold in his book *Why Your Child is Hyperkinetic* should not be carried out without medical supervision (*ibid* 1975, **16**(45), 39).

The authors cited above now report a study of 79 boys, 56 of whom were hyperkinetic but of at least average intelligence and free from convulsive disorder or other neurological disease. On the strength of the entries in a dietary questionnaire filled in by the parents, each child's weekly intake of food additives was rated as high, medium or low on an empirical scale. No significant differences in dietary ratings appeared between the hyperkinetic boys and the controls. Neither was there any significant difference in additive consumption between the 44 hyperkinetic boys who were being treated with methylphenidate or imipramine and the 12 who were not undergoing treatment. The weight gain among the medicated boys over a 12-month period was lower, however, probably because of the known anorectic effect of the drugs. There is no support in this analysis for the hypothesis of Feingold, that hyperactivity is associated with ingestion of unusually high doses of food additives. The form of the study did not permit consideration of the suggestion that there may be a genetically controlled predisposition to hypersensitivity to additives.

AGRICULTURAL CHEMICALS

3055. Cart before the horse for dibromochloropropane?

Rosenkranz, H. S. (1975). Genetic activity of 1,2-dibromo-3-chloropropane, a widely-used fumigant. *Bull. Env. contam. & Toxicol. (U.S.)* **14,** 8.

The usefulness of short-term bacterial mutagenesis tests in the assessment of carcinogenic hazard has been discussed at some length (*Cited in F.C.T.* 1976, **14.** 353). Positive results in these bacterial systems may indicate the need for a well-designed long-term

carcinogenicity test in a mammalian species, but the situation was reversed with 1,2-dibromo-3-chloropropane (DBCP) in that animal testing had indicated carcinogenic properties (*ibid* 1975, **13,** 144) but microbial studies had not been reported. Short-term bacterial tests have now been undertaken on this compound to determine further their reliability in predicting carcinogenicity.

DBCP, a widely used fumigant and nematocide, inhibited the growth of *Escherichia coli* pol A_1^- (a strain deficient in DNA polymerase I) but had no effect on *E. coli* pol A^+ (a strain with the normal complement of this enzyme), a finding indicative of reaction between DBCP and cellular DNA, upon which carcinogens are thought to act. DBCP was also shown to be mutagenic to strain TA1530 of *Salmonella typhimurium* but unreactive to strain TA1538 of the same micro-organism. This indication that it induced base-substitution but not frameshift mutations in the 'Ames' bacterial system (*ibid* 1975, **13,** 465 & 466) suggested that DBCP acted as an alkylating agent.

[For the proponents of short-term bacterial screening procedures for carcinogens, the results of this study are gratifying. However, it must be recognized that a similar short study indicating no such correlation between carcinogenicity and mutagenicity would probably not have been worth publishing. The debate thus continues.]

FEED ADDITIVES

3056. Another profile of terephthalate toxicity

Moffitt, A. E., Jr., Clary, J. J., Lewis, T. R., Blanck, M. D. & Perone, V. B. (1975). Absorption, distribution and excretion of terephthalic acid and dimethyl terephthalate. *Am. ind. Hyg. Ass. J.* **36,** 633.

Terephthalic acid (TA) and its dimethyl ester (DMT) are used industrially in polymer production and as intermediates in adhesives, herbicides, printing inks, coatings and paints; TA is also added to some animal feed supplements to increase the bioavailability of antibiotic additives. It has been reported that rats fed 1% DMT for 96 days showed reduced body weight, although lower proportions of DMT in the feed were without significant effect, and that inhalation of fine DMT dust had no adverse effect (*Cited in F.C.T.* 1975, **13,** 156).

In the study cited above, more than 80% of ^{14}C-labelled TA or DMT given orally to rats was recovered from the urine and faeces within 48 hr. TA was evenly distributed between urine and faeces, whereas DMT appeared predominantly in the urine, less than 10% being recovered in the faeces. No appreciable accumulation of either compound occurred when oral doses were repeated on five alternate days. After intratracheal injection in rats, a single dose of [^{14}C]TA was excreted mainly (about 66%) in the urine, with a little (6%) in the faeces within 48 hr, and a single dose of [^{14}C]DMT was distributed similarly, the total recovery being some 62%. Less than 1% of the TA or DMT remained in the lungs and tracheal nodes 24 hr after the injection. Single or repeated applications of 80 mg [^{14}C]TA to the shaved skin of rats resulted in negligible absorption, but 11% of labelled DMT similarly applied was excreted in the urine and faeces after a single dose and 13% after doses on five alternate days. Neither compound caused any irritation at the site of application.

After application of 50 mg TA or DMT to the conjunctival sac of one eye of each rabbit for 5 min or 24 hr, no significant absorption of TA was detected, but 29 and 37% of the dose of DMT appeared in the urine after the 5-min and 24-hr applications, respectively.

The evidence suggests that although TA and DMT may be absorbed rapidly by some routes, excretion is equally rapid and no tissue accumulation occurs after single or repeated doses.

PROCESSING AND PACKAGING CONTAMINANTS

3057. Suspicion falls on the adipates

Singh, A. R., Lawrence, W. H. & Autian, J. (1975). Dominant lethal mutations and antifertility effects of di-2-ethylhexyl adipate and diethyl adipate in male mice. *Toxic. appl. Pharmac.* **32,** 566.

The use of several plasticizers in the polymer industry has recently been examined critically in view of their possible adverse effects on man. Up to the present time, attention has been concentrated on the reported embryotoxicity and teratogenic potential of the phthalates in rodents (*Cited in F.C.T.* 1972, **10,** 723; *ibid* 1975, **13,** 587). The adipates, which are used predominantly as plasticizers in PVC destined for low-temperature applications, have also been shown to be teratogenic to rats (*ibid* 1974, **12,** 784). The authors cited above now report the effects of the diethyl and di-2-ethylhexyl adipates (DEA and DEHA) on the reproduction of the mouse.

DEHA was injected ip into male mice at levels of 0·5, 1·0, 5·0 and 10 ml/kg, and the more toxic DEA was administered similarly at levels of 0·44, 0·73, 1·10 and 1·46 ml/kg. Each treated animal was immediately caged with two virgin female mice, the latter being replaced weekly for 8 wk. The resulting pregnant mice were killed on day 15 ± 2 of gestation. Effects on reproduction were evaluated with respect to the whole 8-wk experimental period and in relation to wk 1–3 and 4–8 after the administration of the adipates, wk 1–3 representing sperm in the postmeiotic stage of development at the time of injection and wk 4–8 representing sperm in the premeiotic stage.

Both adipates exhibited antifertility effects. The highest dose of DEHA significantly reduced the percentage of pregnancies (67% compared with 82% in controls) whereas DEA showed a reduction at the two higher dosages (59 and 60% compared with 71% in controls). A dose effect was demonstrated statisti-

cally over the postmeiotic period in the DEA-treated groups. The overall mean number of implantations/pregnancy was not affected by either ester. However, DEA-treated animals showed a significant reduction (both time- and time and dose-dependent) in the number of implantations in the premeiotic stage, whereas DEHA injection produced a time-dependent and dose-dependent reduction during the postmeiotic stage. Early foetal deaths, a direct measure of dominant lethal mutations, were increased in the DEHA-treated groups in both post- and premeiotic stages. The DEA situation was more complex but, overall, a dosage effect could be demonstrated. The final reproductive parameter investigated was the number of live foetuses in each pregnancy. A significant effect of both dose and time was demonstrated during the postmeiotic stage in the DEHA-treated mice, and of both time and time-dosage during the premeiotic stage in the DEA-treated mice.

Thus, the two adipates tested reduced fertility and exhibited mutagenic effects on the reproductive system of the mouse. These effects were generally, but not exclusively, associated with the postmeiotic stage of spermatogenesis.

THE CHEMICAL ENVIRONMENT

3058. Following environmental arsenic

Lakso, J. U. & Peoples, S. A. (1975). Methylation of inorganic arsenic by mammals. *J. agric. Fd Chem.* **23,** 674.

Bettley, F. R. & O'Shea, J. A. (1975). The absorption of arsenic and its relation to carcinoma. *Br. J. Derm.* **92,** 563.

The resurgence of arsenic (As) in the environment as a result of its use as a herbicide in forestry and as an animal feed supplement to improve meat yield has given rise to some disquiet (*Cited in F.C.T.* 1975, **13,** 285). Further information regarding the environmental chemistry of As therefore comes as a welcome addition to the earlier studies.

The ability of certain strains of Methanobacterium to methylate inorganic As, and the observation that methylated As compounds are present in mammalian urine and in plants, prompted the first study cited above. Four cows were fed sodium arsenate ($Na_2HAsO_4.7H_2O$) in five consecutive daily doses of 2·75 mg/kg and urine samples were collected over the 3 days before, 5 days during, and 7 days after administration. After a further 7 days, the same cows were fed potassium arsenite ($KAsO_2$) in five daily doses of 1·57 mg/kg, urine samples being collected as before. Four male dogs were fed doses of 3·40 mg arsenate/kg and 1·94 mg arsenite/kg in the same manner.

None of the test animals showed signs of intoxication or had any garlic odour indicative of As poisoning. The control levels of methylated As and inorganic As in the cow urine were 0·12–0·26 and 0·05–0·17 ppm, and these rose to peaks of 3·53 and 1·33 ppm, respectively, during arsenate feeding, and 4·78 and 1·57 ppm, respectively, during arsenite feeding. For dogs, the control values were 0·20–0·31 and 0·11–0·16 ppm with peaks of 6·23 and 10·48 ppm for arsenate feeding and 5·03 and 5·16 ppm for arsenite feeding, respectively. In both species, control levels of methylated and inorganic As were regained within 5 days of the end of feeding. A short delay in the increased excretion of methylated As after arsenite feeding suggests that oxidation to arsenate may precede alkylation. The percentage of As excreted as methylated As by dogs, although less than that excreted by cows, indicates that the rumen cannot be the sole site of methylation (as was thought likely owing to its intense anaerobic bacterial activity). The authors suggest that methylation is a detoxication mechanism for inorganic As.

The possible role of As retention in the production of keratoses and carcinoma of the skin in carcinoma-prone individuals was investigated in the second study cited above. Four patients with arsenical carcinoma were given a total dose equivalent to 8·52 mg As in three doses at 8-hr intervals. Both the As concentration in the blood and total As excretion in the urine were lower in these patients than in control subjects who received the same doses. Determinations of As excretion in faeces were inconclusive, no more than 3·5% of the dose being excreted by this route. Among men, no significant difference in the As content of beard shavings could be detected between the two groups. Between 3·5 and 6·5 mg of the total dose administered could not be accounted for by excretion, the carcinoma-prone patients appearing to retain up to twice as much As in their tissues as did the controls. The moral of these findings is that occult and environmental sources of As may well be relevant to the development of carcinoma in susceptible persons though not in resistant persons with better powers of excretion. From the therapeutic standpoint, it is possible that if carcinoma-prone patients could be identified, others might be treated with As for psoriasis without expectation of undesirable side-effects.

3059. Arsenic and angiosarcoma

Lander, J. J., Stanley, R. J., Sumner, H. W., Boswell, D. C. & Aach, R. D. (1975). Angiosarcoma of the liver associated with Fowler's solution (potassium arsenite). *Gastroenterology* **68,** 1582.

Industrial exposure to inorganic arsenic has been correlated with an increased incidence of lung cancer, and sometimes also of lymphatic and skin cancer. Skin cancer has also developed after chronic ingestion of Fowler's solution for the treatment of psoriasis or asthma (*Cited in F.C.T.* 1972, **10,** 101). Angiosarcoma of the liver, which is now an established hazard of vinyl chloride exposure (*ibid* 1976, **14,** 347) has in only one previous case been associated with prolonged ingestion of Fowler's solution (Regelson *et al. Cancer, N.Y.* 1968, **21,** 514), but other cases have involved three of 27 German vineyard workers exposed to arsenical pesticides (Roth, *Z. Krebsforsch.* 1957, **61,**

468) and the inhabitant of an arsenic-rich area of South America (Rennke *et al. Revta méd. Chile* 1971, **99**, 664). A second case resulting from prolonged treatment with Fowler's solution is now reported.

A 43-yr-old man, who had ingested 10–15 drops of Fowler's solution daily from 1951 to 1966 for treatment of psoriasis, was admitted to hospital in 1972 with melaena (black blood in the faeces) and subsequent haemoperitoneum (bleeding into the peritoneal cavity). Laparotomy revealed a large cystic mass involving the right lobe of the liver, which had lacerated in its inferior border. The laceration was sutured, but the patient returned 2 months later after vomiting blood. The oesophageal veins were found to be swollen and distorted, and selective angiography of the liver demonstrated features consistent with both hepatoma and cavernous haemangioma. Hyperkeratotic papules were evident on soles and palms, and the legs were hyperpigmented. Despite radiation therapy the tumour spread to the left lobe of the liver, haemoperitoneum recurred, and a small erythematous papule which developed on the patient's cheek was found to be an angiosarcoma. Death occurred 1 yr after initial hospital admission. Autopsy revealed that the liver tumour was an angiosarcoma composed of malignant cells lining vascular spaces which invaded and almost completely obliterated the hepatic parenchyma. Metastatic nodules were found in the lungs. However, there was no evidence of liver fibrosis or of cirrhosis, which occurred in 13 of the 27 German vineyard workers (Roth, *loc. cit.*) and in three non-alcoholic patients treated with Fowler's solution (Franklin *et al. Am. J. med. Sci.* 1950, **219**, 589). The authors emphasized the long latent period for the development of such malignancies (21 yr in this case) and suggested that additional cases might occur as a result of the previous widespread use of Fowler's solution.

3060. Cadmium and metallothionein

Chen, R. W., Whanger, P. D. & Weswig, P. H. (1975). Biological function of metallothionein. I. Synthesis and degradation of rat liver metallothionein. *Biochem. Med.* **12**, 95.

Metallothionein (MT) is known to bind cadmium (Cd) in the kidney (*Cited in F.C.T.* 1975, **13**, 471), and there is some evidence that it plays a part in the detoxication of heavy metals. It has also been suggested that MT may be involved in the metabolism of essential trace metals (Evans, *Nutr. Rev.* 1971, **29**, 195; Webb, *Biochem. Pharmac.* 1972, **21**, 2751), and that the synthesis of defective MT may be implicated in some diseases, notably Wilson's disease (Evans *et al. Science N.Y.* 1973, **181**, 1175). The authors cited above investigated the synthesis and degradation of Cd-stimulated MT to establish more clearly its biological role.

Since it was known that incorporation of [^{14}C]cystine into liver MT was very low in unexposed rats, but was greatly increased by an injection of Cd, rats were injected sc with 26·7 μmol $CdCl_2$/kg and 3 days later were given an ip injection of [^{14}C]cystine in a dose of 20 μCi/kg. The animals were killed at various times from 1 hr to 11 days after this latter injection and the soluble fraction of the liver homogenate was chromatographed on a Sephadex G-75 column and subjected to scintillation counting. In one series of studies, the effects of injected mercury, silver, zinc and copper on the synthesis of MT were compared with the effect of Cd.

Using these procedures, it was shown that the single injection of a Cd salt caused a fourfold increase in the incorporation of labelled cystine into MT. The ^{14}C label first appeared in the MT fraction 2 hr after its administration. The stimulatory effect of a Cd injection on levels of ^{14}C in MT had two time peaks, at 5 hr and at 6 days. The amount of Cd in the MT fraction increased to a maximum after 4 days and then remained constant for the rest of the experiment (11 days). Cd injection appeared also to cause accumulation of zinc in MT; the zinc level reached a peak 4 days after Cd injection, but then, unlike Cd, it decreased with time. The finding that zinc disappeared from the MT fraction at a rate similar to that of [^{14}C]MT, suggested an involvement of MT in zinc metabolism. This was supported by a demonstration that an injection of zinc sc also stimulated MT synthesis, a property shared by injected copper and mercury, but not by silver under the experimental conditions used.

In degradation studies, rat-liver MT was found to have a half-life of 4·2 days. This did not differ greatly from the half-life of 4·9 days found after a second Cd injection had been given 1 day after the cystine administration. The similarity in the two figures suggested that MT was not stabilized by Cd. In view of the apparent involvement of MT in zinc metabolism, it was thought possible that MT was stabilized by zinc rather than by Cd.

On the basis of the persistence of Cd in tissues, it had been assumed that MT had a very long half-life, but the present study indicated the fallacy of this argument, since MT was shown to have a turnover rate similar to those of other liver proteins. The question then arose of how Cd remained constant in the MT fraction while MT was degraded. Of several possibilities being investigated, the most likely seemed to be that Cd released from degraded MT stimulated further MT synthesis and was bound to the newly formed, and hence unlabelled, protein. This was supported by the long-lasting stimulatory effect of Cd on MT synthesis. The authors of this paper suggest that MT is a zinc-storage protein in which the levels may be influenced by other metals, such as Cd, mercury and copper.

3061. Nickel binding still to be unravelled

Hutchinson, F., Macleod, T. M. & Raffle, E. J. (1975). Nickel hypersensitivity. Nickel binding to amino acids and lymphocytes. *Br. J. Derm.* **93**, 557.

Nickel is one of the most common causes of contact dermatitis (*Cited in F.C.T.* 1976, **14**, 217) and has been said to be responsible for more reactions of this type than all other metals combined (Fisher, *Contact Dermatitis*, 2nd ed., p. 96, Lea & Febiger, Philadelphia, 1973). This allergic response has frequently been

investigated by the patch-test method, but recently several workers have utilized the lymphocyte transformation test (LTT) when seeking a more sensitive technique for the detection of contact hypersensitivity (*Cited in F.C.T.* 1973, **11,** 699; *ibid* 1974, **12,** 576). The former study demonstrated that both nickel sulphate and nickel acetate specifically increased transformation in blood samples from sensitive individuals but gave no clues as to the nature of the reaction between the nickel and the lymphocytes. In the study cited above, radiochromatographic and autoradiographic techniques were used to elucidate the binding of nickel in the transformation test.

Preliminary two-way thin-layer cellulose chromatography with Medium 199 containing ^{63}Ni-labelled nickel chloride indicated that the nickel had been bound to glycine, histidine and lysine and, to a lesser extent, to aspartic acid and serine. Quantitative assay, involving liquid scintillation counting of the individual amino acid spots, revealed lysine as the major binding acid.

A solution containing ^{63}Ni was also added to cultures of both control and nickel-sensitive lymphocytes suspended in Medium 199 or in phosphate-buffered saline with or without the addition of autologous serum. Autoradiographs of the samples demonstrated binding of ^{63}Ni to approximately 20% of lymphocytes from both normal and nickel-sensitive subjects. This was observed with cells suspended in Medium 199, in serum or in phosphate-buffered serum.

These findings suggest two possible triggering methods for lymphocyte transformation: conjugation of nickel with an amino acid (mainly lysine) in the culture medium, and direct binding to the surface of the lymphocyte cell, as was observed with 20% of lymphocytes from both normal and nickel-sensitive subjects irrespective of the supporting medium. The latter observation indicates that binding is not by itself the transformation stimulus. A third mechanism involving hapten-protein conjugation on the lymphocyte surface is also postulated.

3062. Metal sensitivity from joint replacements

Elves, M. W., Wilson, J. N., Scales, J. T. & Kemp, H. B. S. (1975). Incidence of metal sensitivity in patients with total joint replacements. *Br. med. J.* **4,** 376.

Dermatitis in patients fitted with stainless-steel prostheses has been attributed to nickel leaching into the tissues (*Cited in F.C.T.* 1976, **14,** 216). Other metals may also play a role, and skin tests with nickel, cobalt and chromium salts in 14 patients with loose metal-on-metal prostheses revealed positive reactions in nine; no evidence of sensitivity was found, however, in 24 patients with stable prostheses (Evans *et al. J. Bone Jt Surg.* 1974, **56B,** 626).

When 50 patients who had received total joint replacements (mostly of the metal-on-metal variety) were patch-tested with nickel, cobalt, chromium, molybdenum, titanium and vanadium, 19 (38%) gave positive reactions to one or more of the metals. Two reacted to nickel only, eight to cobalt only, six to both nickel and cobalt, one to both nickel and vanadium and two to chromium. In three of the 50 patients, the appliance was fractured. Of 23 patients in whom non-traumatic failure of the prosthesis had occurred, as many as 15 (65%) were sensitized, whereas of the 24 with stable prostheses only four gave positive reactions (three to nickel and cobalt and one to cobalt alone). Two of these patients had a previous history of metal-contact sensitivity. The total incidence of sensitivity in patients with non-loosened prostheses, including the three whose appliances had failed because of trauma, was only 15%.

Dermatitis occurred after the operation in 13 patients, eight of whom were later found to be sensitive to metal salts. In three of these there was a previous history of metal sensitivity. Onset of dermatitis varied from 1 wk to 24 months after operation, and the condition normally resolved itself after a few days, but in one case it lasted some months and in another secondary infection occurred, necessitating amputation.

None of the patients sensitive to cobalt alone had a history of metal sensitivity, and the incidence of cobalt sensitization (28%) was much greater than that previously reported among patients attending contact dermatitis clinics (about 1·5%), strongly suggesting that sensitivity was correlated with prosthesis wear. In contrast, six of the nine nickel-sensitive patients and one of two chromium-sensitive patients had a previous history of metal dermatitis. The possibility that development of metal sensitivity may be the cause, rather than the effect, of prosthesis wear is now being investigated in a further study.

3063. *N*-Ethyl-*N*-methylaniline metabolism

Gorrod, J. W., Temple, D. J. & Beckett, A. H. (1975). The metabolism of *N*-ethyl-*N*-methylaniline by rabbit liver microsomes: The measurement of metabolites by gas-liquid chromatography. *Xenobiotica* **5,** 453.

Gorrod, J. W., Temple. D. J. & Beckett, A. H. (1975). The differentiation of *N*-oxidation and *N*-dealkylation of *N*-ethyl-*N*-methylaniline by rabbit liver microsomes as distinct metabolic routes. *Xenobiotica* **5,** 465.

Previously reported metabolic studies on *N*-alkylanilines have used either expensive radiotracer techniques or unsatisfactory non-specific colorimetric methods. The two studies cited above investigated the metabolism of *N*-ethyl-*N*-methylaniline (EMA) in the rabbit by a new gas–liquid chromatographic technique. EMA was incubated in stoppered flasks with various rabbit-tissue homogenates, and the alkylanilines produced, either directly or by subsequent conversion of any EMA *N*-oxide formed, were extracted with ether. The extracts were then analysed chromatographically, using a column capable of separating all the possible volatile metabolic products of EMA, namely *N*-methylaniline, *N*-ethylaniline and aniline, and the unchanged substrate.

Monodealkylation of EMA was shown to occur almost exclusively in the lungs and liver, and *N*-oxidation was greatest in the lungs and liver, although there was significant activity in the kidney and blad-

der. The subcellular distribution of metabolizing enzymes in the lung and liver was investigated, and conversion was shown to occur predominantly in the microsomal fraction of both organs. In spite of the relatively high pulmonary enzyme activity (about half that of the liver), only a low level of cytochrome *P*-450 was observed in this tissue fraction. The relative rates of the metabolic transformations of the liver microsomes were: *N*-demethylation > *N*-oxidation > *N*-de-ethylation.

The second paper describes the further investigation of the metabolic pathway of EMA in the liver microsomes. Although NADP was a necessary cofactor for optimum conversion rates in the microsomal incubation, NADH was also shown to be effective, producing half of the optimum rate. Addition of NAD or NADH to a standard incubation mixture (i.e. one already containing NADP) caused a 30% increase in dealkylation but did not affect *N*-oxidation. Although the exact significance of these results was uncertain they did suggest that dealkylation and *N*-oxidation proceeded by two distinct routes, a point supported by inhibitor studies. Standard inhibitors of cytochrome *P*-450, such as SKF 525-A, decreased the rate of *N*-dealkylation but not of *N*-oxidation and inhibitors of the microsomal electron transport chain, such as *n*-propyl gallate, produced a similar effect, suggesting that separate electron transport chains were involved in dealkylation and in oxidation.

The authors discuss briefly the possible mechanism of the dealkylation of EMA. Previous workers had postulated that dealkylation proceeded through an *N*-oxide intermediate (Bickel, *Pharmac. Rev.* 1969, **21,** 325). In the present study, however, no secondary amines were detected when EMA *N*-oxide was incubated with the hepatic microsomal fraction, demonstrating that this route was not available for EMA. The study also indicated that ring hydroxylation was not a major factor in EMA metabolism *in vitro*.

3064. An alternative fate for formate in the rat

Palese, M. & Tephly, T. R. (1975). Metabolism of formate in the rat. *J. Toxicol. envir. Hlth* **1,** 13.

The role of metabolism in methanol poisoning is well documented, but although *in vivo* oxidation of methanol (*Cited in F.C.T.* 1970, **8,** 109) and its probable metabolite formaldehyde (*ibid* 1974, **12,** 160) has been extensively investigated, the *in vivo* oxidation of formate, a possible agent for the toxicity of methanol, has not received the same attention.

Early studies *in vitro* demonstrated a direct relationship between liver catalase activity, which liberates oxygen from hydrogen peroxide, and formate oxidation. More recent investigations have suggested that the folate-dependent one-carbon pool is implicated in the oxidation, the formate being activated prior to its entry into the many reactions of the pool (*ibid* 1966, **4,** 100). The authors cited above attempted to determine the relative importance of these systems in the metabolism of formate in the rat.

Effectively two groups of rats were used for the investigations *in vivo*—one in which the animals were fed a diet deficient in folic acid and a control group given a normal diet. The former were also regularly treated with succinylsulphathiazole to inhibit folic acid synthesis by the gut flora. After 6–8 wk on these dietary regimes, the liver folate content of the rats on the deficient diet was a third of that of the control animals. Sodium [^{14}C]formate was administered to the rats by ip injection; the resulting $^{14}CO_2$ was collected in a caustic soda trap, and the radioactivity was determined by a liquid scintillation technique.

In control rats, the rate of oxidation of formate was concentration-dependent up to a maximum rate, which occurred at doses above 500 mg/kg. The ip injection of either 3-amino-1,2,4-triazole (AT) 1 hr before the sodium formate, or of ethanol simultaneously with the sodium formate, had no effect on the rate of $^{14}CO_2$ production. AT and ethanol, however, did inhibit formate oxidation by liver homogenates from the control animals, and glycollic acid, which increases hydrogen peroxide production, is known to stimulate the production of $^{14}CO_2$ in this system. As both AT and ethanol are well known inhibitors of the catalase-peroxidative system, it seems that this system was involved in the oxidation of formate by the rat tissues *in vitro* but not *in vivo*.

The rate of oxidation of sodium formate in the folate-deficient rats was only approximately half that in the controls, but after 3–4 wk on a folate-supplemented diet the oxidation rate returned to the control levels. When AT or ethanol was administered to the folate-deficient animals there was a further marked decrease in formate oxidation. These results suggest that catalase played a significant role in formate metabolism in the rat *in vivo* only when the folate-dependent one-carbon pool was depleted; under normal conditions the more efficient folate pathway was preferred.

The study demonstrated that rats, which unlike rhesus and pigtail monkeys do not suffer from methanol acidosis, have an alternative pathway available for the oxidation of formate. If the catalase route is important in primates, formate oxidation would be slow because of the relatively low levels of hepatic peroxide-generating enzymes present in these species. The contribution of formic acid to the acidosis of methanol poisoning in the monkey is currently under investigation.

3065. Carcinogenic potential for HMPA?

Zapp, J. A., Jr. (1975). HMPA: a possible carcinogen. *Science, N.Y.* **190,** 422.

Hexamethylphosphoramide (HMPA) has been widely used as a laboratory solvent and insect chemosterilant. Its acute toxicity after ingestion, inhalation and skin absorption is low to moderate, but its long-term toxicity is more severe. In rats HMPA has caused testicular atrophy and lung fibrosis (*Cited in F.C.T.* 1967, **5,** 820; *ibid* 1969, **7,** 269), and consistent effects on the fertility of male rats given doses of 100 mg/kg or more have led to the demonstration of its antispermatogenic potency (*ibid* 1971, **9,** 303).

An inhalation study of HMPA in rats now indicates that this compound possesses distinct carcinogenic potential. Rats were exposed to up to 4000 ppb

(b = 10^9) HMPA for 6 hr daily on 5 days/wk. In rats killed after 6 months, no lesions were detected. Exposures for 6–8 months resulted in an increase in deaths among animals breathing 400 or 4000 ppb HMPA, the main cause of death being degenerative disease of the kidney tubules. Some rats killed after 8 months had enlarged noses and experienced breathing difficulty as a result of squamous cell carcinoma which originated in the epithelium of the turbinate bones and which, in some animals, filled the nasal cavity and penetrated into the brain. Of the 240 animals exposed to 4000 ppb HMPA, 12 of the 15 that died or were killed at 8 months had nasal tumours, and seven of the eight in the group of 240 exposed to 400 ppb HMPA for the same period had similar tumours. A random selection of six rats exposed to 50 ppb HMPA showed no malignancy at this stage. The observations clearly indicate that the carcinogenic potential of HMPA requires closer examination.

3066. Polychlorinated biphenyls under the microscope

Bickers, D. R., Eiseman, J., Kappas, A. & Alvares, A. P. (1975). Microscope immersion oils: effects of skin application on cutaneous and hepatic drug-metabolizing enzymes. *Biochem. Pharmac.* **24,** 779.

Polychlorinated biphenyls (PCBs) have become widespread in the environment (*Cited in F.C.T.* 1973, **11,** 131; *ibid* 1975, **13,** 574). Like their relatives, the polychlorinated terphenyls, PCBs have been shown to be powerful inducers of liver microsomal enzymes in birds and to cause hepatic hypertrophy in rabbits after percutaneous absorption (*ibid* 1973, **11,** 134). The presence of PCBs in microscope immersion oils therefore presents a potential hazard, which has prompted the paper cited above.

In the USA, microscope immersion oils may contain 30–45% PCBs. Two samples were diluted with mineral oil to reduce viscosity and applied daily in equivalents of 1 or 10 μl immersion oil to the shaved skin of rats for 6 days. In a further experiment, a single application of 10 μl was made, and the rats were examined at intervals thereafter. Both oils, applied at 10 μl/day for 6 days, markedly increased liver weight, microsomal protein and NADPH cytochrome *c* reductase. Cytochrome *P*-450 and ethylmorphine *N*-demethylase activity increased three- or four-fold and benzo[*a*]pyrene hydroxylase increased 15- to 20-fold. There was a marked increase in the 455:430 nm peak ratio of the ethyl isocyanide difference spectrum of the reduced microsomes. Application of the lower dose produced significant though less marked effects, except that induction of benzo[*a*]-pyrene hydroxylase was similar to that following the higher dose and NADPH cytochrome *c* reductase was not increased significantly. Induction of hepatic cytochrome *P*-448 and alteration of the haemoprotein spectrum were similar to changes induced by polycyclic hydrocarbons. Significant enhancement of the same parameters occurred after the single application of 10 μl of oil, the response reaching a maximum after 2 days and lasting for at least 10 days. Such findings indicate the need for further studies to evaluate the effects of immersion oils on skin hydroxylase activity in man.

3067. Breaking up polymer glycols

Haines, J. R. & Alexander, M. (1975). Microbial degradation of polyethylene glycols. *Appl. Microbiol.* **29,** 621.

The biodegradation of synthetic polymers is of considerable environmental importance, particularly in view of their increasing use for food packaging. The degradation of polyethylene by ultraviolet irradiation followed by attack by soil micro-organisms has been reported from Sweden (*Packaging* 1974, no. 526, p. 13) and the ability of soil-derived organisms to deal with glycol polymers is illustrated in the paper cited above.

The biological oxygen demand (BOD) method was applied to determinations of the biodegradability of mono-, di-, tri- and tetraethylene glycols and of polyethylene glycols (PEG) in the molecular weight range 400–20,000. All the test compounds were degraded by a strain of *Pseudomonas aeruginosa* and a number of uncharacterized bacteria derived from soil samples. Ethylene glycol was substantially degraded within 2 days, the higher glycols up to PEG 400 within 5 days, and PEGs up to 10,000 within 20 days. In addition, *P. aeruginosa* was able to attack PEG 20,000, and a supernatant from a culture grown in medium containing PEG 20,000 gave a compound identified by gas chromatography as diethylene glycol. An extracellular-enzyme preparation derived from this organism produced ethylene glycol as a metabolite of PEG 20,000. The authors discuss possible degradation routes and conclude that extracellular enzymatic hydrolysis of the polymer was followed by utilization of a low-molecular-weight metabolite, possibly ethylene glycol, as a carbon and energy source by the organism.

[The use of selected cultures of *Pseudomonas aeruginosa* as a polymer-disposal method might present some problems, since many strains of this organism are aggressive and troublesome pathogens for man.]

3068. Incompatibility of ethanol and TCE

Müller, G., Spassowski, M. & Henschler, D. (1975). Metabolism of trichloroethylene in man. III. Interaction of trichloroethylene and ethanol. *Arch. Tox.* **33,** 173.

Trichloroethylene (TCE) is metabolized in man via the transient chloral hydrate to trichloroethanol, thought to be the toxic metabolite, and trichloroacetic acid (*Cited in F.C.T.* 1976, **14,** 158), the reactions being mediated by enzyme systems which, in part, also control the oxidation of ethanol. It is well known that ethanol potentiates the effect of TCE on the central nervous system, the liver and the kidney, and the authors cited above investigated the mechanism of TCE–ethanol incompatibility.

A single 6-hr exposure of volunteers to TCE at an atmospheric level of 100 ppm was combined with administration of ethanol in a dose giving a mean

blood level of 0·6%. Observations on these subjects were compared with those recorded 4 wk earlier in the same volunteers exposed to TCE under comparable conditions without concurrent ethanol administration. The presence of ethanol caused at least a twofold increase in TCE in the blood and a threefold increase in TCE in the expired air. Concurrent determination of trichloroethanol in the blood and urine showed that the concentration was more than halved by ethanol treatment and that formation of trichloroacetic acid was completely inhibited while ethanol was present in the body. This latter observation was attributed to inhibition of chloral hydrate oxidation by the reduction in NAD level known to be associated with ethanol oxidation, since the enzyme, chloral hydrate dehydrogenase, is largely NAD-linked.

The authors conclude that, in contrast to the normal effect of TCE, which is mediated by the toxic metabolite, trichloroethanol, ethanol increased the level of unmetabolized TCE in the blood and hence in the brain, leading to TCE levels in the central nervous system approaching the subhypnotic concentration.

3069. A wash-out for flame retardants

Gutenmann, W. H. & Lisk, D. J. (1975). Flame retardant release from fabrics during laundering and their toxicity to fish. *Bull. env. contam. & Toxicol.* (*U.S.*) **14,** 61.

The organophosphorus compounds tris-(2,3-dibromopropyl) phosphate (DBP), *N*-methyloldimethyl phosphonopropionamide (Pyrovatex CP) and tetrakis(hydroxymethyl)phosphonium hydroxide (THPOH) have been used as flame retardants in textiles. The toxicity of these to goldfish (*Carassius auratus*) at 1 ppm in water has now been studied. After 5 days of exposure to DBP, all the fish died following a period of disorientation; no abnormal behaviour appeared in fish exposed to Pyrovatex CP for 30 days, and the toxicity of THPOH was of intermediate severity.

In order to determine the extent to which these compounds might be released during washing, treated fabrics were boiled with water to simulate a laundering process. For each compound the fabrics yielded up to 10 μg/sq in., the amount depending on the type of fabric and the thickness of the retardant finish, and similar quantities of retardant were liberated during a second and third washing operation on the same sample. The phosphate present in the washings was demonstrated to be of organic origin and was, therefore, not due to traces of inorganic phosphorus in the fabric. It was calculated that washing the equivalent of six domestic sheets in a total volume of 30 gallons of water would, if they were treated with these organophosphorus retardants, result in a combined wash- and rinse-water concentration of up to 6 ppm. The addition of detergents might aggravate this situation, but the presence of untreated fabrics might reduce the concentration by reabsorption. The extent to which laundry water is purified in a sewage system and its degree of dilution when discharged would be important factors in determining the possible environmental hazard of flame-proofing treatment.

NATURAL PRODUCTS

3070. Aflatoxin and alveolar cell carcinoma

Dvořáčková, I. (1976). Aflatoxin inhalation and alveolar cell carcinoma. *Br. med. J.* **1,** 691.

Aflatoxin produces cancer of the liver, stomach, intestine and kidney in the rat when administered orally (*Cited in F.C.T.* 1969, **7,** 377) and has been associated tentatively with cases of primary liver cancer in man (*ibid* 1976, **14,** 152). It has also been reported to cause squamous-cell carcinoma in the lung when instilled into the trachea of rats (*ibid* 1966, **4,** 638), and has now been suspected of causing alveolar-cell carcinoma in a man who inhaled dust containing the mould *Aspergillus flavus.*

A 68-yr-old chemical engineer developed a severe pulmonary disease 3 months after completing a 3-month period of work on a method of sterilizing Brazilian peanut meal contaminated by *A. flavus.* The disease affected first the left lung and later both. The patient died 11 months after the onset of his illness and at autopsy the lungs were found to be enlarged and to contain numerous firm yellowish-white or reddish lesions. Histologically the lesions were made up of fibrous tissue and of irregular alveoli lined with cuboidal or columnar epithelium in place of the usual simple squamous epithelium. Multinucleate giant cells were present within the alveoli. Chemical analysis of the lung tissue revealed the presence of aflatoxin B_1. A colleague who had been doing the same work as this patient for an unspecified period had died 3 yr earlier of the same malignant condition, but no chemical investigations had been made.

The authors suggest that persons inhaling toxic moulds during their work might be at risk from this disease.

[The time between termination of exposure and the onset of symptoms is too short in this case to support the suspicion of an aetiological relationship between aflatoxin and alveolar-cell carcinoma. These tumours usually progress slowly in their early stages and it seems likely that the disease was already present when the 68-yr-old engineer commenced his project on the sterilization of Brazilian peanuts.]

3071. Metabolism of palmotoxin

Bassir, O. & Emerole, G. O. (1975). Species differences in the metabolism of palmotoxins B_0 and G_0 *in vitro*. *Xenobiotica* **5,** 649.

Palmotoxins B_0 and G_0, isolated from *Aspergillus flavus*, have been shown to produce liver lesions in the chick embryo resembling those produced by the aflatoxins. Palmotoxin B_0 has also exhibited hepatic toxicity similar to that of aflatoxin B_1 when administered ip to rats, but in the same study palmotoxin G_0 produced no comparable toxic effects (*Cited in F.C.T.* 1975, **13,** 677).

Continuing their studies of the palmotoxins, the authors named above have investigated the metabolism of these compounds in the liver fractions of eleven animal species, including the mundane rat, albino mouse, guinea-pig, rabbit, dog, duck and toad and the more exotic dwarf sheep and goat, white rock cock and rainbow lizard.

Liver slices (200–500 μm) and microsomal liver fractions (10,000 **g** supernatant) of each of these species were incubated with palmotoxin B_0 or G_0 and the metabolic products were separated by thin-layer chromatography. In general, the total metabolic conversion of the palmotoxins was far lower than that of the aflatoxins; typically 40–60% of the palmotoxin was metabolized, whereas 75–99% conversion of aflatoxin B_1 occurred under comparable conditions.

The sheep and rabbit showed the greatest ability to metabolize both palmotoxin substrates, whereas the guinea-pig exhibited the lowest metabolic capacity. All species except the cock and duck produced a hydroxylated fluorescent metabolite at levels of 0·2–1·2% from both palmotoxins, and the rat alone produced a second fluorescent hydroxylated metabolite from palmotoxin B_0. Formaldehyde production, indicative of demethylation, was comparatively high in the rat, mouse and dog but did not occur in either the duck or goat.

The authors are clearly aware of the experimental limitations of this preliminary study, but the results do indicate substantial differences between the metabolism of the palmotoxins and aflatoxins. Avian species have been found to metabolize the aflatoxins more readily than other species, but in the present work the duck and rock cock were not particularly active in their metabolism of the palmotoxins and produced no hydroxylated derivative. Toxicological data are not available to enable any correlation between susceptibility of species and rates of metabolism to be made, but it seems probable that the toxic effects of the palmotoxins do not depend on conversion of the parent compounds to metabolites.

3072. The remote chance of lanolin allergy

Clark, E. W. (1975). Estimation of the general incidence of specific lanolin allergy. *J. Soc. cosmet. Chem.* **26,** 323.

Lanolin is the name commonly given to anhydrous lanolin, wool fat or wool wax, a complex mixture of esters of high molecular weight alcohols and fatty acids. It is generally agreed that the allergen of lanolin, which is a weak one and which defies attempts to predict its activity, resides in the alcohol fraction (*Cited in F.C.T.* 1970, **8,** 474). In view of the widespread occurrence of lanolin in cosmetic preparations it has been suggested that manufacturers should be obliged to indicate its presence by appropriate labelling.

The review paper cited above states that 0·6% lanolin is deliberately retained in wool for textile manufacture in order to preserve the softness of the fibre. This concentration is as high as that in many cosmetics and causes most people to come into prolonged contact with lanolin whether or not they apply it cosmetically. Nevertheless, no case of lanolin hypersensitivity has come to light among at least 3000 workers handling raw wool or refining the derived wax over a period of 28 yr. Although no single component has been identified as the allergen, polyvalent hypersensitization often includes sensitivity to lanolin, and the author emphasizes the need to distinguish between primary lanolin allergy and secondary lanolin hypersensitivity. Recent data based on a population of 825,000 served by skin clinics in Gothenburg, Lund and Wycombe indicate that the incidence of specific lanolin allergy does not exceed 9·7/million, and is probably considerably less.

3073. Methylxanthines and the hepatic processing enzymes

Aeschbacher, H.-U. & Würzner, H.-P. (1975). Effect of methylxanthines on hepatic microsomal enzymes in the rat. *Toxic. appl. Pharmac.* **33,** 575.

The activity of the liver processing enzymes acetanilide hydroxylase and *o*-nitroanisole demethylase was increased in rats by administration of three oral doses of 40 mg caffeine/kg/day (*Cited in F.C.T.* 1968, **6,** 674). This activity was suppressed by inhibitors of protein synthesis, suggesting that caffeine acted by inducing the synthesis of microsomal enzymes, but attempts to verify this hypothesis produced somewhat equivocal results (*ibid* 1972, **10,** 105). Elsewhere it has been reported that rats ingesting 20–24 mg caffeine/kg/day in their drinking-water for 8 wk showed only a temporary increase in acetanilide hydroxylase activity accompanied, however, by a marked depression in aminopyrine *N*-demethylase activity (Khanna & Cornish, *Fd Cosmet. Toxicol.* 1973, **11,** 11). The effects of caffeine and other methylxanthines in this connexion are considered further in the paper cited above.

Rats were orally intubated with caffeine, theophylline or theobromine in a daily dose of 150 mg/kg for 3 days, with caffeine at 75 mg/kg for 6 days, or with the three methylxanthines singly or in combination, each at a level of 37·5 mg/kg, for 6 days. The rats were killed 3, 12 or 48 hr after the last dose, and aliquots of each liver-microsomal fraction were incubated for 30 min with aniline, with aminopyrine and with *p*-nitroanisole.

At 150 mg/kg, all three methylxanthines significantly increased aniline hydroxylase activity, theobromine being the most potent in this respect and theo-

phylline the least, although none was as potent as phenobarbitone (60 mg/kg), used as a positive control. Aminopyrine demethylation was increased by theobromine and theophylline, and the former also stimulated *p*-nitroanisole demethylase activity. Caffeine given at 75 mg/kg for 6 days increased the activity of aniline hydroxylase and of aminopyrine demethylase, despite its lack of significant effect on this enzyme at twice the dose for half the time. However, at 37·5 mg/kg none of the methylxanthines had a significant effect on aniline hydroxylase (the most sensitive of the three enzymes), even when all were given together in a total dose of 112·5 mg/kg, suggesting that each acts individually on the microsomal enzymes.

Enzyme induction, which was not apparent 3 hr after any treatment, was maximal 48 hr after the 3-day 150-mg/kg treatments and 12 hr after the 6-day lower-dose schedules. Relative liver weight was increased by 150 mg caffeine/kg, but the content of liver microsomal protein was not correlated with enhancement of enzyme activity by any of the methylxanthines, indicating a lack of action on enzyme synthesis.

The authors calculate that the dose of 37·5 mg caffeine/kg would be equivalent to 25 cups of coffee or tea daily, and since much lower amounts of theobromine and theophylline are found in these beverages an effect on the human liver processing enzymes is thought unlikely.

[It has, however, already been shown that lower dose levels may be active in this respect when administered for 8 wk (Khanna & Cornish, *Fd Cosmet. Toxicol.* 1973, **11,** 11) and the effects of longer-term administration still remain to be clarified.]

3074. The role of pterosins and pterosides in bracken toxicity

Saito, M., Umeda, M., Enomoto, M., Hatanaka, Y., Natori, S., Yoshihira, K., Fukuoka, M. & Kuroyanagi, M. (1975). Cytotoxicity and carcinogenicity of pterosins and pterosides, 1-indanone derivatives from bracken (*Pteridium aquilinum*). *Experientia* **31,** 829.

Shikimic acid has been identified as one of the carcinogenic factors in bracken (*Cited in F.C.T.* 1975, **13,** 405) and a second fraction of the plant extract has been shown to possess more potent carcinogenicity than shikimic acid, although it appears to have no appreciable embryotoxicity or teratogenicity in mice (*ibid* 1975, **13,** 481).

The study cited above is concerned with sesquiterpenes containing the 1-indanone nucleus (pterosins) and their glycosides (pterosides), prepared from the methanol extract of dried young bracken fronds, and their effect upon HeLa cells grown in Eagle's medium supplemented with calf serum. Cell injuries sustained after contact with the test compound for 3 days were assessed in five categories ranging from insignificant to lethal. Morphological changes with intermediate toxicity levels involved reduction in cell size and the production of some spindle-shaped cells with a clear, sometimes vacuolated, cytoplasm. The nuclei showed a deeper staining with haematoxylin and a scatter of chromatin condensates, while the nucleolus became rounded and mitotic figures were decreased. Pterosin B, abundant in bracken fronds, was tested for chromosomal effects by incubation with HeLa cells at 100 and 32 μg/ml for 24 or 48 hr; the mitotic index fell but no morphological breakage was apparent. Pterosin B was found to inhibit the cellular uptake of ^{3}H-labelled DNA, RNA and protein precursors. Neither the quinic acid nor the shikimic acid fractions of an ethyl acetate extract of bracken demonstrated cytotoxicity.

Feeding dried edible parts of bracken as 30% of the diet of Wistar rats was associated with a 19% incidence of intestinal tumours after some 420 days, while the same feed proportion of dried rhizome was followed by a 44% incidence of such tumours. Benzene or methanol extracts of bracken fronds, but not pterosin B fed at 4 mg/day or pteroside B fed at 10 mg/day, were also associated with a high incidence of tumours in rats.

3075. The dusty problem of nasal cancer

Ironside, P. & Matthews, J. (1975). Adenocarcinoma of the nose and paranasal sinuses in woodworkers in the state of Victoria, Australia. *Cancer, N.Y.* **36,** 1115.

Nasal adenocarcinomas are known to be a hazard for woodworkers making furniture in Europe, and it has been suggested that α,β-unsaturated aldehydes in wood lignins may be the compounds responsible (*Cited in F.C.T.* 1971, **9,** 899; *ibid* 1974, **12,** 289).

From a study of medical statistics, the authors cited above suggest that heavy exposure to wood dust in the cutting and working of Australian native timbers may amount to a carcinogenic hazard. Among 99 cases of malignant tumours of the nose or paranasal sinuses recorded in the Cancer Institute at Melbourne, 19 were adenocarcinomas. The patients with this tumour included only one woman, and a search for some occupational factor revealed an association with woodworking in seven of the 13 patients whose occupation could be traced. From a further series of 80 patients suffering from other types of malignant nasal or sinus tumours, only four were singled out as having had an association with woodworking. Unlike the workers in Europe, however, who are mainly at risk from the fine dust produced in the furniture industry, the Australian workers included carpenters and sawmillers exposed to coarser wood dust.

COSMETICS, TOILETRIES AND HOUSEHOLD PRODUCTS

3076. Short-term fate and long-term effects of hair dyes

Maibach, H. I., Leaffer, M. A. & Skinner, W. A. (1975). Percutaneous penetration following use of hair dyes. *Archs Derm.* **111,** 1444.

Wernick, T., Lanman, B. M. & Fraux, J. L. (1975). Chronic toxicity, teratologic, and reproduction studies with hair dyes. *Toxic. appl. Pharmac.* **32,** 450.

It has been shown that the percutaneous absorption of hair-dye compounds is usually low (*Cited in*

F.C.T. 1972, **10,** 884) and more recently demonstrated that little if any metabolic alteration of a specific dye occurred during *in vivo* transit (*ibid* 1975, **13,** 163). The first paper cited above reports the percutaneous penetration potential and fate of two commercially distributed semipermanent hair dyes in man, rats and rabbits.

As a result of reports from several women that their urine darkened after hair dyeing, two of the semipermanent dyes involved (A and B) were applied to the scalp hair of eight adult male volunteers. Separation of the dye constituents by thin-layer chromatography had shown the presence of at least nine component chemicals in each of the two dyes. In order to determine the penetrative potential of the dyes, 24-hr urine specimens were collected daily for 5 days. After lyophilization and extraction of the urine samples, a purple spot was detected on the chromatograms of the urine extracts of four individuals whose hair was dyed with either dye A or dye B. This purple metabolite, not present in the extracts of control urines, was excreted 1–4 days after application of either dye. A faint purple spot, corresponding to that of the human metabolite, was also observed in three rats treated with dye B but, as in the human studies, the amounts of the metabolite collected were insufficient for identification. In a similar experiment on one rabbit treated with dye B, no purple metabolite could be detected.

From these preliminary studies, the authors were unable to state whether the dye component giving rise to this metabolite was stored, slowly metabolized and excreted, or whether it penetrated only slowly into the skin. They suggested that identification of individual dye components would assist in future studies.

In some earlier work (Burnett *et al. Fd Cosmet. Toxicol.* 1975, **13,** 353) on three oxidation hair dyes, which are oxidized to compounds of a higher molecular weight under normal conditions of use, no signs of systemic toxicity or carcinogenicity were observed. The dyes involved in the second study cited above were of the semipermanent type, mainly nitrophenylenediamines and nitroaminophenols and their *N*-substituted derivatives. These semipermanent compositions are merely adsorbed by the hair and are available for absorption through the skin of the scalp.

A composite formulation of a series of semipermanent hair dyes and base components was administered in the diet to dogs at levels providing intakes of 0·0, 19·5 and 97·5 mg/kg/day over a period of 2 yr. During this time the animals produced no evidence of adverse effects on growth, haematological values, liver-function tests, organ weights or histopathology. The urine excreted by all animals of the test group was blue-brown in colour but was otherwise normal.

Groups of male and female rats fed with the composite at concentrations of 0, 1950 and 7800 ppm in the basal diet showed no significant dose-related differences in male or female fertility, length of gestation, number of females with resorption sites, live pups per litter, pup body weights or pup survival. Female rats fed with these dietary levels of composite from day 6 to day 15 of gestation showed no significant dose-related differences in the mean number of implantation sites, live pups or early and late resorptions per litter or in the number of females with one or more resorption sites. No evidence of any teratological effect was seen in these animals or in rabbits dosed daily, by gavage, on days 6–18 of gestation with either the composite at doses of 19·5 or 97·5 mg/kg/day or the composite base without the dyes (97·5 mg/kg/day).

Average human usage of hair dyes is a monthly application of a formulation containing 2–5% total dye in a volume of 2 ml/kg. The 5% level of dye is thus equivalent to 100 mg/kg which is of a considerably lower order than the high-level oral dose of 97·5 mg/kg/day administered in this study.

[A more direct approach, investigating the metabolic fate and possible toxic effects of the individual dye components, rather than composites, would certainly clarify the situation. This strategy, also suggested by the authors of the first of the above papers, has been adopted by some workers (*Cited in F.C.T.* 1975, **13,** 163).]

METHODS FOR ASSESSING TOXICITY

3077. Food allergy testing compared

Soifer, M. M. & Hirsch, S. R. (1975). The direct basophil degranulation test and the intracutaneous test: a comparison using food extracts. *J. Allergy clin. Immun.* **56,** 127.

The direct basophil degranulation (DBD) test has been compared with the intracutaneous (IC) test in 14 individuals with a history of sensitivity to specific foods and in a control group consisting of nine individuals without a history of allergic symptoms and five others sensitive to inhalant allergens but not to food constituents. Of 17 DBD tests carried out on the food-allergic subjects, 15 showed significant degranulation. These same subjects also gave a positive IC test result. When 13 of them were tested on 16 occasions with food extracts to which they were not sensitive, there were 13 occasions on which a negative IC test was associated with a lack of significant degranulation, and only one on which a positive IC test accompanied a negative DBD test. Among the 29 comparisons in the control subjects there were 24 where the IC test and DBD test were both negative and three where both tests were positive, while in two tests on a single subject the IC test was negative and the DBD test positive. The overall correlation between the results of the DBD and IC tests was 92%, between the IC test and the history of allergy 90% and between the DBD test and the history of allergy 86%.

The high correlation between the two tests and the history of the patient is acknowledged to be due possibly to patient selection and the use of fresh allergens. Further evaluation of the DBD test as a diagnostic aid for hidden food allergy is therefore suggested.

3078. No simple test for skin irritancy

Nixon, G. A., Tyson, C. A. & Wertz, W. C. (1975). Interspecies comparisons of skin irritancy. *Toxic. appl. Pharmac.* **31,** 481.

Results of irritancy determinations on rabbit skin using modifications of the Draize test (*Toxic. appl. Pharmac.* 1971, **19,** 276) do not always correlate well with those of tests on human skin; nevertheless comparative tests have confirmed the rabbit as a suitable animal for preliminary irritancy testing, since in general it was more sensitive than other test animals (*Cited in F.C.T.* 1973, **11,** 346). Further reflections on the problems of inter-species comparison arise from the paper now under review.

Fifteen household products and nine industrial chemicals were tested for irritancy on intact and abraded skin of rabbits, guinea-pigs and man by a 4-hr application. Isopropyl alcohol, sodium tripolyphosphate, 10% sulphuric acid and table salt gave insignificant reactions in all test species. High- and low-carbonate detergents, phosphate detergent, enzyme detergent, liquid detergent, liquid cleaner, soap, antiperspirant, lemon juice and sodium carbonate produced slight to moderate irritation in rabbits, guinea-pigs or both, but not in man. Pine oil cleaner, liquid shampoo and sodium lauryl sulphate were slightly irritant to human skin and moderately irritant to rabbits, guinea-pigs or both. Three materials produced more severe reactions in man than in the test animals: hypochlorite bleach, slightly irritant in rabbits and guinea-pigs, caused eschar and weeping on the intact skin of four of seven human subjects, fatty acids (C_8–C_{10}) caused slight reactions in guinea-pigs, moderate to severe erythema in rabbits and weeping and eschar formation on the intact skin of two of seven humans. and metasilicate/carbonate detergent produced severe erythema and vesication of intact human skin and severe necrosis in rabbits, but a negligible reaction in guinea-pigs.

Much discrepancy between human and animal observations arose when abraded skin sites were tested, and it is recommended that such tests should be discontinued to avoid unnecessary confusion in interpretation. It is recommended, moreover, that neither the rabbit nor the guinea-pig should be relied upon exclusively in testing for potential human-skin irritants.

PATHOLOGY

3079. Cloudy swelling clarified?

Ling, G. N. & Walton, C. L. (1976). What retains water in living cells? *Science, N.Y.* **191,** 293.

According to the classical theory, intracellular water is retained by means of an intact cell membrane, while the water content is regulated by a variety of complex biochemical 'pumps', which need ATP to function. A second hypothesis suggests, however, that an undamaged membrane is not essential to the retention of water within the cell. Support for this view has come from some recent studies on the frog sartorius muscle.

Under carefully controlled conditions, centrifugation of the intact muscle for 4 min at a relative centrifugal force varying between 400 and 1500 **g** extracted a constant volume of tissue water equal to the volume of the extracellular fluid in the muscle. The volume of fluid removed in this way was found to be the same even when the cell membranes had been damaged by a series of carefully placed incisions in the muscle tissue, which consists of parallel, elongated cells each about 3 cm long. Furthermore, intact and cut muscle tissue swelled to the same degree when immersed in a hypotonic solution.

These experiments, and others involving inhibition of tissue metabolism, suggest that the integrity of the cell membrane is not essential for the retention of cell water. It is thought that intracellular water is adsorbed on to cell proteins, with ATP functioning not as a 'fuel' but in a key physico-chemical role. This involves its attachment to critical sites on the protein molecule, which is thereby maintained in an extended conformation allowing the backbone peptide groups to polarize, orient themselves and retain deep layers of water.

[This new concept is of some interest to the pathologist. Minor accumulation of intracellular water, called 'cloudy swelling', cannot be accounted for satisfactorily by the old hypothesis, since biochemically and ultrastructurally there is little evidence of breakdown of cell components. The new hypothesis suggests that cloudy swelling could be due to minor conformational changes in the protein molecule.]

CANCER RESEARCH

3080. Bladder tumours from nitrosomethyldodecylamine

Lijinsky, W. & Taylor, H. W. (1975). Induction of urinary bladder tumors in rats by administration of nitrosomethyldodecylamine. *Cancer Res.* **35,** 958.

A study of 65 different nitrosamines revealed that asymmetric dialkylnitrosamines are generally oesophageal carcinogens (*Cited in F.C.T.* 1968, **6,** 648). This study included a variety of *N*-methyl-*N*-alkylnitrosamines of chain length up to C_7 (*n*-heptyl-). For symmetrical dialkylnitrosamines, the primary site of action is typically the liver, although dibutylnitrosamine is also a potent bladder carcinogen (*ibid* 1965, **3,** 498). The latter effect is attributed to the hydroxylated metabolite, butyl-(4-hydroxybutyl)nitrosamine which, when administered to mice in the drinking-water, produced lethal bladder tumours without the appearance of other toxic effects—in males much ear-

lier than in females (*ibid* 1974, **12,** 251). In the paper cited above, the asymmetric nitrosomethyl-*n*-dodecylamine (NMDA) is reported to display a pattern of toxicity similar to that of the dibutylnitrosamine metabolite.

NMDA was administered to rats by gavage, twice weekly for 50 wk, at a level of 12 mg in olive oil. The animals were then allowed to die naturally or were killed when moribund. All rats died 48–80 wk after the start of treatment, except one, which was killed at wk 96. Females tended to survive longer than males, despite their lower body weight in proportion to the dose level. All the 30 rats so treated developed transitional cell carcinomas of the bladder, accompanied in seven cases by alveologenic adenomas of the lung but in only one case by an oesophageal tumour, in contrast to the effects of asymmetric dialkylnitrosamines reported earlier. Metastases from bladder to lung also occurred in four animals. The bladder tumours, which appeared to arise from multiple foci in the urinary mucosa, had in some areas differentiated into adenocarcinomas with acinar formation and into squamous-cell carcinomas with keratin formation. An absence of tumours from a similar group of rats given the tertiary amine from which the NMDA was prepared, suggested that an impurity in the NMDA was unlikely to have been responsible for the demonstrated tumorigenicity, since it was also present in the precursor. The authors can offer no explanation for their unexpected findings.

[The only obvious distinction between the asymmetric dialkylnitrosamine tested here and those previously shown to induce oseophageal tumours is the marked difference in the length of the alkyl chain.]

Fd Cosmet. Toxicol. Vol. 14. p. 519. Pergamon Press 1976. Printed in Great Britain

LETTER TO THE EDITOR

BACTERIAL MUTAGENICITY AND CARCINOGENIC POTENTIAL

Sir,—The reliability of bacterial mutagenesis as a potential indicator of mammalian cancer risk is debatable, but there is no doubt that a large number of proven carcinogens give positive findings for mutagenicity. It is also interesting to consider the results of mutagenicity tests for compounds with a doubtful status in terms of carcinogenic potential and, in particular, for the halogenated aliphatic hydrocarbons.

McCann *et al.* (*Proc. natn. Acad. Sci. U.S.A.* 1975, **72**, 5135) reported that carbon tetrachloride gave negative results for mutagenic activity, and Uehleke *et al.* (Vth Annual Meeting of the European Environmental Mutagen Society, Firenze, 19–22 October 1975, p. 70) confirmed this and also recorded negative findings for base-pair and frame-shift mutation with chloroform. Both of these haloalkanes have been reported to produce hepatomas when administered at a dose level sufficient to cause severe liver injury (Eschenbrenner & Miller, *J. natn. Cancer Inst.* 1944, **5**, 251; *idem, ibid* 1946, **6**, 325). The response in the liver to hepatotoxic dose levels of carbon tetrachloride has been claimed to be related to hyperplasia, rather than to neoplasia directly attributable to the compound (Confer & Stenger, *Cancer Res.* 1966, **26**, 834), and, of course, this is not the only instance of nodular hyperplasia following the administration of hepatotoxic compounds (Grasso *et al. Fd Cosmet. Toxicol.* 1969, **7**, 425). A clear distinction between the carcinogenicity of a substance and the production of tumours by repeated tissue injury and regeneration is not universally accepted, but the difference could be crucially important if substantiated, since a carcinogen may be capable of initiating tumours when given at a low dose level whereas the risk of producing tumours in the course of tissue damage and repair is obviously confined to dose levels sufficient to cause substantial necrotic change. True carcinogens would seem more likely to act as mutagens under the usual conditions of bacterial mutagenicity testing than substances whose particular attribute is their gross toxicity to mammalian cells. Thus, the negative mutagenicity findings for carbon tetrachloride and chloroform are consistent with the view that they are not carcinogenic

The position of vinyl chloride in this connexion is not clear, mainly because the present bacterial mutagenicity tests are not easily adapted for testing volatile compounds. Scanning the data of McCann *et al.* (*loc.cit.*) on about 300 chemicals for those giving 'positives' for carcinogenesis and 'negatives' for mutagenesis, it was not easy to find compounds for which dose–response data in long-term mammalian studies were readily available. An interesting instance, however, is safrole; this compound, reported as being negative in mutagenicity testing, is hepatotoxic and 'carcinogenic' at high dose levels (Hall, in *Toxicants Occurring Naturally in Foods*; National Academy of Sciences, Washington, D.C., 1973, p. 448), but has not been reported to yield an excess of neoplasms at lower exposure levels.

Compounds with indisputable carcinogenic potential usually show a relatively shallow dose–response curve for tumour initiation. In cases where a long-term dose–response experiment suggests a steep curve, there has previously been some reluctance to accept the idea of a threshold dose level for tumours, usually because the numbers of animals at each dose level have been relatively modest. However, negative bacterial mutagenicity findings may be of considerable value in helping to clarify the distinction between toxic and carcinogenic potential.

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Fd Cosmet. Toxicol. Vol. 14, p. 521. Pergamon Press 1976. Printed in Great Britain

MEETING ANNOUNCEMENT

ENVIRONMENTAL MUTAGENS

The Second International Conference on Environmental Mutagens, to be held in Edinburgh on 11–15 July 1977, will be concerned with the mutagenic effects of environmental agents, particularly as they represent a hazard to man.

Plenary lectures will deal with the relationship between mutagenesis, carcinogenesis and teratogenesis, the involvement of repair in the mutation of mammalian cells, genetic health protection and the relative contribution of mutations, chromosome abnormalities and non-disjunction to genetic ill-health in man.

A series of symposia and sessions for contributed papers will cover all aspects of this area of research.

Registration forms and further information may be obtained from The Secretary General: Dr. P. Brookes, Institute of Cancer Research, Nightingale Lane, Chalfont St. Giles, Bucks., HP8 4SP. The closing date for the submission of presentations is 31 December 1976.

Fd Cosmet. Toxicol. Vol. 14, p. 523. Pergamon Press 1976. Printed in Great Britain

FORTHCOMING PAPERS

It is hoped to publish the following research papers and other communications in the next issue of *Food and Cosmetics Toxicology*:

Acute (mouse and rat) and short-term (rat) toxicity studies on Erythrosine BS. By K. R. Butterworth, I. F. Gaunt, P. Grasso and S. D. Gangolli.

Short-term toxicity of Erythrosine BS in pigs. By K. R. Butterworth, I. F. Gaunt, P. Grasso and S. D. Gangolli.

Studies on the absorption, distribution and excretion of citral in the rat and mouse. By J. C. Phillips, J. Kingsnorth, S. D. Gangolli and I. F. Gaunt.

Effects of butylated hydroxytoluene on *Tetrahymena pyriformis*. By J. G. Surak, R. L. Bradley, Jr., A. L. Branen, W. E. Ribelin and E. Shrago.

Influence of dietary nitrate on nitrite content of human saliva: possible relevance to *in vivo* formation of *N*-nitroso compounds. By B. Spiegelhalder, G. Eisenbrand and R. Preussmann.

The effect of nitrate intake on nitrite formation in human saliva. By. S. R. Tannenbaum, M. Weisman and D. Fett.

Dimethylnitrosamine formation in the gastro-intestinal tract of rats. By S. Hashimoto, T. Yokokura, Y. Kawai and M. Mutai.

Studies on pancreatic islet accumulation and cytotoxicity of *N*-nitrosomethylurea in the cynomolgus monkey. By H. Tjälve, E. Wilander and E.-B. Johansson.

Some effects of dietary citric acid in small animals. By E. Wright and R. E. Hughes.

Tannic acid: acute hepatotoxicity following administration by feeding tube. By. A. Oler, M. W. Neal and E. K. Mitchell.

Mycotoxicosis produced in mice by cultural products of an isolate of *Aspergillus ochraceus*. By J. L. Zimmermann, W. W. Carlton and J. Tuite.

Comparative metabolism of dieldrin in the rat (CFE) and in two strains of mouse (CF1 and LACG). By D. H. Hutson.

Histochemical and biochemical studies of chemically induced acute kidney damage in the rat. By R. C. Cottrell, C. E. Agrelo, S. D. Gangolli and P. Grasso.

2,3,7,8-Tetrachlorodibenzo-*p*-dioxin elimination in the rat: first order or zero order? By R. A. Hiles and R. D. Bruce. (Short Paper).

Monographs on fragrance raw materials. By D. L. J. Opdyke.

Contents continued]

Aims and Scope

The Journal provides a wide-ranging service of general articles and abstracts on food and cosmetics toxicology. The Journal is primarily intended for the use of food scientists and technologists, manufacturers and administrators who neither read nor have access to the medical, pharmacological or toxicological literature. In addition, the journal will constitute a medium for the publication of reviews and original articles relating to the fields of interest covered by the British Industrial Biological Research Association.

Instructions to Authors

General. Authors from the United Kingdom should send *Original Papers and Reviews* to the Assistant Editor. All other papers and reviews should be sent to the appropriate Regional Editor. All 'Letters to the Editor' should be sent to the Editor and must be signed before they can be considered for publication.

Submission of a paper to the Editor will be held to imply that it reports unpublished original research, that it is not under consideration for publication elsewhere and that if accepted for the Journal, *Food and Cosmetics Toxicology*, it will not be published again, either in English or in any other language, without the consent of the Editor.

Forms of Papers Submitted for Publication. Papers should be headed with the title of the paper, the surnames and initials of the authors and the names and addresses of the institutions where the work was done. A shortened version of the title not exceeding 45 letters and spaces, to serve as a running title, should be supplied.

In general the text should be subdivided as follows: (a) Summary: brief and self-contained, not exceeding 3% of length of paper (b) Introductory paragraphs (c) Experimental (d) Results, presented as concisely as possible (e) Discussion (if any) and general conclusions (f) Acknowledgements and (g) References. Each subdivision should commence on a separate sheet. Manuscripts should be typewritten on *one side of the paper* and *double spaced*. At least *two copies* should be supplied (one original and one, or preferably two, carbon copies). Papers will be accepted in English, French and German.

References. These should be listed on a separate page, in alphabetical order and arranged as follows: author's name and initials, year, title of the journal [abbreviated according to the rules adopted in the *World List of Scientific Periodicals 1963*, 4th Ed., Butterworth & Co. (Publishers) Ltd. London], volume, first page number:

e.g. Hickman, J. R., McLean, D. L. A. & Ley, F. J. (1964). Rat feeding studies on wheat treated with gamma-radiation. I. Reproduction. *Fd Cosmet. Toxicol.* **2**, 15.

References to books should include the author's name followed by initials, year, title of book, edition, appropriate page number, publisher and place of publication:

e.g. Dow, E. & Moruzzi, G. (1958). *The Physiology and Pathology of the Cerebellum*. 1st Ed., p. 422. The University of Minnesota Press, Minneapolis.

The names of all the authors of papers to be cited should be given when reference is first made in the text. In cases where there are more than two authors subsequent citations should give the first-named author followed by the words *et al.*:

e.g. (McLaughlin, Bidstrup & Konstam, 1963); (McLaughlin *et al.* 1963).

Where more than one paper by the same author(s) has appeared in any one year, the references should be distinguished in the text and the bibliography by the letters, a, b etc. following the citation of the year:

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Footnotes. These as distinct from literature references should be avoided as far as possible. Where they are essential, reference is made by the symbols * † ‡ § ‖ ¶ in that order.

Illustrations and Diagrams. These should be kept to a *minimum* and they should be numbered and marked on the back with the author's name. Legends accompanying illustrations should be typewritten on a *separate* sheet. Diagrams and graphs must be drawn in Indian ink on good quality paper or tracing linen. The following standard symbols should be used on line drawings since they are easily available to the printers:

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Other Nomenclature, Symbols and Abbreviations. In general, authors should follow the recommendations published in the *Handbook for Chemical Society Authors* (1961), p. 164 and in the *I.U.P.A.C. Information Bulletin*, No. 13, p. 64, Appendix B (1961). In the title and summary, abbreviations should be avoided; in the Introduction, Results and Discussion they should be used sparingly.

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