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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

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Research Section

SHORT-TERM TOXICITY STUDY OF ALLYL CAPROATE IN RATS

S. A. CLODE, K. R. BUTTERWORTH, I. F. GAUNT, P. GRASSO* and S. D. GANGOLLI

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(*Received 13 September 1977*)

Abstract—Groups of 15 male and 15 female rats were given daily oral doses of 0 (control), 35 or 100 mg allyl caproate/kg/day, as a solution in oil, for 14 wk, and ten males and ten females were given 12 mg/kg/day for 13 wk. There were no differences between treated and control rats in body weight, water intake, haematological examinations, analysis of urine, renal concentration tests or serum chemistry. There were slight increases in food intake at the highest dose level. The weight of the liver was increased with doses of 35 and 100 mg/kg/day, and all treated groups showed evidence of periportal vacuolation, which was dose-related in both incidence and severity and which, in the rats given 100 mg/kg/day, was accompanied by enlargement of the hepatocytes, focal periportal necrosis and bile-duct proliferation. Weights of the kidneys, spleen, stomach and small intestine were increased at the highest level of treatment. The small-intestine weight was also increased in females given 35 mg/kg/day. On the basis of these findings it was not possible to establish a no-untoward-effect level for allyl caproate.

INTRODUCTION

Allyl caproate ($CH_3 \cdot [CH_2]_4 \cdot COO \cdot CH_2 \cdot CH: CH_2$; allyl hexanoate; 2-propenyl hexanoate) is a clear colourless oil used as a constituent of flavourings in beverages, chewing-gum, ice-cream, sugar confectionery and flour confectionery. In the Report on the Review of Flavourings in Food prepared by the Food Additives and Contaminants Committee (1976), allyl caproate is included in the list of flavourings regarded as provisionally acceptable for use, subject to a limit of 200 ppm in food, although evidence of enzymatic hydrolysis is required. It is classified by the Council of Europe (1974) among flavouring substances that may be added temporarily to foodstuffs without hazard to public health, proof of enzymatic hydrolysis again being requested together with acute and short-term studies, and it has been classed as 'generally recognized as safe' by the Flavoring Extract Manufacturers' Association (1965). The data supplied to us by seven leading flavouring manufacturers suggest a maximum level of consumption in all foods of 24·56 mg/day, which is equivalent to an intake of 0·35 mg/kg/day by a 70-kg adult.

There is some information on the metabolism of allyl caproate. Longland, Shilling & Gangolli (1977) showed that the ester was hydrolysed rapidly in the intestine to yield allyl alcohol. This is supported by the work of Butterworth, Carpanini, Gaunt, Grasso & Lloyd (1975), who compared the development of hepatic periportal necrosis following administration of equimolar doses of allyl alcohol and some of its esters. The extent of this effect was similar with the alcohol and the caproate ester, suggesting a role for allyl alcohol in the toxicity of the ester. A review of the metabolism and toxicity of allyl alcohol by Carpanini, Gaunt, Hardy, Gangolli, Butterworth & Lloyd (1978) showed that the formation of mercapturic acid was a likely route of metabolism and suggested acrolein as the probable hepatotoxic intermediate. These workers administered allyl alcohol to rats in the drinking-water for 15 wk and detected no hepatic effect in animals given up to 800 ppm (providing an intake of approximately 90 mg/kg/day). This contrasted with the known hepatotoxic effects of similar doses of allyl alcohol given by intubation (e.g. 50 mg/kg; Reid, 1972). It was postulated that the intake of a series of small doses, as occurs with administration in the drinking-water, fails to provide sufficiently high blood concentrations to cause hepatic damage.

Jenner, Hagan, Taylor, Cook & Fitzhugh (1964) found that the oral LD_{50} for allyl caproate was 218 mg/kg in rats, and a short-term study by Hagan, Hansen, Fitzhugh, Jenner, Jones, Taylor, Long, Nelson & Brouwer (1967) showed that daily oral doses of 100 mg/kg given to rats for 18 wk produced some liver damage. In their long-tern studies, however, Hagan *et al.* (1967) found that 2500 ppm (125 mg/kg body weight/day) for 1 yr had no adverse effects on rats, although F. Bär (personal communication 1966) found liver necrosis when allyl caproate was administered to rats for 1·5 yr at a dietary level of 1000 ppm (50 mg/kg body weight/day).

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The present paper describes a short-term toxicity study in rats treated with allyl caproate by oral intubation as part of a safety evaluation programme for flavouring agents.

EXPERIMENTAL

Materials. The sample of allyl caproate was supplied by Bush Boake Allen Ltd., London, and conformed to the following specification: Purity, min. 98·0%; specific gravity (20°C), 0·886–0·890; free acid, max 0·1% (as caproate). The corn oil was supplied by J. Sainsbury Ltd., London.

Animals and diet. Rats of the Wistar strain, obtained from a specified-pathogen-free breeding colony were caged in groups of five, in a room maintained at 21 ± 1°C with a relative humidity of 50–60%. They were fed Spillers' Laboratory Small Animal Diet and water *ad lib.*

Loss from diet. Diets containing 0·2 or 0·4% allyl caproate were prepared and samples of these were exposed to the air in rat-feeding pots in an animal room for 24 and 48 hr. After exposure, the diets were extracted with methanol and the content of allyl caproate was estimated by gas–liquid chromatography. The concentrations of allyl caproate in the exposed diets were compared with those of samples kept in air-tight containers. There was a 30–50% loss from the diets at 24 hr and 60% loss by 48 hr. Because of this loss and as the material was not sufficiently soluble to be administered in the drinking-water, it was decided to give the allyl caproate by stomach tube as a solution in corn oil.

Experimental design and conduct. Groups of 15 male and 15 female rats were given 0 (control), 35 or 100 mg allyl caproate/kg body weight/day by oral intubation once daily on 7 days/wk for 14 wk. The control animals were given corn oil alone. Additional groups of five male and five female rats were given the same dose levels for 2 or 6 wk. After 1 wk an additional group of ten male and ten female rats from the same batch was included in the study and given 12 mg allyl caproate/kg/day. This treatment was continued for 13 wk so that the rats were the same age at termination as those given the higher dose levels. A group of five males and five females, also started on 12 mg/kg/day at the end of wk 1 was treated for 5 wk to terminate with the groups killed at wk 6.

The rats were weighed initially and then weekly throughout the study and the food and water intakes were measured over a 24-hr period preceding the day of weighing. At the end of the appropriate period, the rats were fasted overnight and killed by exsanguination under barbiturate anaesthesia, and blood samples were collected for haematological examination and serum analysis.

All the blood samples were examined for haemoglobin content, packed cell volume and counts of erythrocytes and total leucocytes, and counts were made of reticulocytes and different types of leucocytes in some samples. The serum was separated and analysed for the content of urea. total protein and albumin and for the activities of glutamic–oxalacetic transaminase, glutamic–pyruvic transaminase and lactic dehydrogenase.

Urine was collected during wk 2 and 5 or 6 and in the final week of treatment and was examined for appearance, microscopic constituents and content of cells, glucose, ketones, bile salts and blood. At the same time, a renal concentration test was carried out in the same animals. This involved measuring the specific gravity and volume of urine produced during a 6-hr period of water deprivation and that produced during a 4-hr period commencing after 16 hr without water. To assess the renal diluting ability, the volume and specific gravity of the urine produced in the first 2 hr after the water load were measured. Urinary cell excretion was estimated by counting the number of cells in urine collected in this latter period.

During the post-mortem examination conducted on each animal, any macroscopic abnormalities were noted, and the brain, heart, liver, spleen, kidneys, stomach, small intestine, caecum, adrenals, gonads, pituitary and thyroid were weighed. Samples of these organs and of the spinal cord, salivary gland, trachea, aorta, thymus, lymph nodes, urinary bladder colon, rectum, pancreas, uterus and skeletal muscles, together with any other tissue that appeared abnormal, were preserved in 10% buffered formalin. Paraffin-wax sections of these tissues were stained with haematoxylin and eosin for histopathological investigation.

RESULTS

No abnormalities were seen in the appearance or behaviour of the rats at any time during the study. There were no statistically significant differences in body weight between the treated and control rats (Table 1), although the males given the highest dose level gained slightly less weight than the controls. Male rats showed no statistically significant differences in food and water intake between the test and control groups, except in food intake during the first day of the study in the group given 12 mg allyl caproate/kg/day, but in the females given 100 mg/kg/day the mean intake of food over the experimental period was higher than that of the controls (Table1). There was a smaller and non-significant increase in the corresponding males.

There were no adverse findings in the results of the haematological examinations (Table 2). The count of total leucocytes was higher in males dosed with 100 mg/kg/day than in controls at wk 14, but although this difference was statistically significant, there was no similar finding in males at any dose level at wk 2 or 6 or in females at any time. There were no differences between treated and control groups in the results of the serum analyses, urinary cell excretion or renal concentration tests. The urine of all rats was free from bile, blood, glucose and ketones, and the concentration of albumin was similar in all groups.

There were no statistically significant variations between the organ weights of control and treated animals at wk 2 or 6, but when the results were expressed relative to body weight, the weights of the livers of the rats on the highest treatment level were significantly higher in both males and females at wk 2 and in females at wk 6. A similarly significant increase was found in the relative weight of the stomach of females given 35 or 100 mg allyl caproate/kg for 2 wk, and although this effect was not found in the males at

Table 1. *Mean body weights and food and water consumption of rats dosed with 0–100 mg allyl caproate/kg for 13 wk*

Dose level (mg/kg/day)	Body weight (g) at day					Food consumption (g/rat/day) at day					Mean food consumption (g/rat/day)	Mean water consumption (ml/rat/day)
	0†	28	42	56	91	0†	28	42	56	91		
Males												
0	128	291	341	374	432	16·2	15·2	14·0	14·1	11·8	14·8	22·7
35	127	297	345	380	435	16·2	17·2	15·8	15·1	13·7	15·4	21·9
100	130	283	326	354	403	16·5	16·0	15·2	14·5	13·3	15·7	23·7
0	174	318	360	390	440	16·2	15·6	16·0	13·5	12·8	14·8	22·7
12	170	309	362	392	445	19·8***	13·0	13·4	12·2	11·8	13·7	21·8
Females												
0	113	190	210	222	240	14·1	12·2	12·1	11·7	12·3	12·3	26·4
35	111	188	212	221	236	15·1	12·4	12·2	11·7	10·0	12·2	22·9
100	112	194	216	230	249	15·7	16·3	15·7	14·0	12·8	14·2**	27·8
0	137	201	217	226	238	13·7	12·3	12·9	10·3	11·9	12·3	26·4
12	133	195	215	223	237	16·2	12·7	12·3	8·9	10·2	11·3	22·3

† First day of dosing.

Values of body weights are the means for groups of 15 animals, except in the case of those given 12 mg/kg/day (ten rats/group); those for the treated groups did not differ significantly (P being $>0{\cdot}05$ by Student's t test) from those of the controls. Values for food and water consumption are the means for three (or two) cages of five animals. Figures marked with asterisks differ significantly from those of the appropriate controls, tests of Wilcoxon (1945) being used for the mean values and of Lord (1947) for the individual figures: $^{**}P < 0{\cdot}01$; $^{***}P < 0{\cdot}001$.

wk 2, or in either sex at wk 6, by wk 14 the stomach weights and relative stomach weights were significantly higher in both males and females on the highest treatment level (Table 3). An increase in liver weights at wk 14 was found in males given 35 mg/kg and in females given 100 mg/kg, but the liver weights relative to body weight showed a significant increase between the treated and control groups in both sexes at both levels of treatment. This increase in relative liver weight was dose-related. The body weights of male rats given 100 mg allyl caproate/kg were markedly lower than those of the controls at wk 14 and this was reflected in a decrease in the weight of the thyroid. Although several other organs showed decreases in weight, the changes were not statistically significant and these effects were not seen in the females at any level. After treatment for 14 wk with 100 mg allyl caproate/kg, marked increases in the absolute and relative weights of the small intestine were found in both sexes. Some increase was also apparent at the 35-mg/kg level. Other differences at this time were confined to the highest dose level, the relative spleen and kidney weights being higher in both males and females of this group. There were significant increases in relative caecal weights at wk 2 in males given 35 mg/kg and at wk 6 in males given 100 mg/kg.

Histopathological examination of the test tissues

Table 2. *Haematological values in rats dosed with 0–100 mg allyl caproate/kg/day for 14 wk*†

Dose level (mg/kg/day)	No. of rats	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
Males										
0	15	15·2	44	8·20	0·35	5·52	20	1	78	1
12	9	15·0	43	8·21	0·40	5·76	—	—	—	—
35	15	14·9	43	7·93	0·40	5·41	—	—	—	—
100	15	14·8	43	7·99	0·39	6·98*	25	0	74	1
Females										
0	15	14·4	42	7·57	0·68	4·22	16	2	81	1
12	10	14·1	41	7·47	—	4·22	—	—	—	—
35	15	14·3	41	7·72	0·63	3·53	—	—	—	—
100	15	14·2	41	7·77	0·74	4·78	19	1	79	1

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

† For 13 wk in the case of the groups given 12 mg/kg/day.

Figures are means for the numbers of rats shown. The value marked with an asterisk differs significantly ($^{*}P < 0{\cdot}05$ by Student's t test) from the control figure. Basophils did not account for more than 0·5% of the leucocytes in any group.

Table 3. *Mean relative organ weights of rats dosed with 0–100 mg allyl caproate/kg/day for 14 wk*†

		Relative organ weight (g/100 g body weight)							
		In males				*In females*			
	Dose level (mg/kg/day)	0	12	35	100	0	12	35	100
	No. of rats examined.....	15	10	15	15	15	9	15	15
Organ	Terminal body weight (g).....	426	434	432	395*	236	235	238	240
Brain		0·45	0·45	0·44	0·48	0·75	0·76	0·76	0·75
Heart		0·25	0·25	0·25	0·26	0·32	0·31	0·31	0·34
Liver		2·51	2·53	2·73*	2·82***	2·63	2·69	2·79*	3·17***
Spleen		0·16	0·15	0·17	0·20*	0·23	0·22	0·23	0·29*
Kidneys		0·51	0·52	0·51	0·56*	0·60	0·57	0·57	0·65*
Stomach		0·42	0·42	0·43	0·49***	0·58	0·58	0·61	0·64*
Small intestine		2·21	2·33	2·39	2·66***	3·05	3·06	3·36**	3·51***
Caecum		0·25	0·25	0·25	0·26	0·33	0·35	0·34	0·37
Adrenals‡		18·6	17·4	16·7	18·3	28·0	28·6	28·9	27·5
Gonads§		0·85	0·83	0·82	0·90	48	45	52	49
Pituitary‡		2·3	2·3	2·0	2·3	4·7	4·5	4·9	4·6
Thyroid‡		4·5	4·5	4·5	4·1	6·2	5·9	6·8	6·4

† For 13 wk in the case of the groups given 12 mg/kg/day.
‡ Weights of these organs are expressed in mg/100 g body weight.
§ Weights of female gonads are expressed in mg/100 g body weight.
Values are means for the numbers of animals shown and those marked with asterisks differ significantly (Student's *t* test) from the control values: $*P < 0.05$: $**P < 0.01$: $***P < 0.001$.

preserved at autopsy revealed a dose-related increase in periportal vacuolation in the liver. The controls showed no comparable changes. When the results of the post-mortem examinations at all time intervals were pooled, it was found that at the lowest dose, 3/15 males and 3/15 females had slight to moderate periportal vacuolation; at the intermediate dose the lesions were more pronounced and affected 2/25 males and 7/20 females, and at the highest dose the lesion occurred in 16/25 males and 11/25 females. Other histological changes were seen in the liver only in rats given the highest dose. These were bile-duct proliferation, enlargement of hepatocytes and focal necrosis in the periportal area. An isolated adenoma of the lung was found in a male dosed with 100 mg allyl caproate/kg/day for 14 wk. The kidneys, heart and lymph nodes showed varying degrees of degenerative change, with a similar incidence in both treated and control animals.

DISCUSSION

Few effects were seen during this study. The reason for the increase in food consumption observed in the animals given the highest dose of allyl caproate (100 mg/kg/day) is unknown, but it was probably associated with the strong taste of the material. A similar response has been encountered in other studies in these laboratories involving the administration of strongly flavoured materials by intubation (Gaunt, Mason, Hardy, Lansdown & Gangolli, 1974). The dose was given during the day when rats do not normally eat and may have stimulated them to consume small amounts of additional food.

Hepatotoxicity and changes in the weights of several organs were seen. The increases in relative weight of the stomach and small intestine in rats given 100 mg/kg for 14 wk were not seen at wk 6, and at wk 2 only the stomach weight of the females was increased. These increases may have been due to a local irritant effect. They occurred mainly at the highest dose level, the administration of which by stomach tube might be expected to cause some irritation of the stomach and probably also of the intestine. However, there were no signs of irritation during the study, such as diarrhoea, and none were seen in the histopathological examination. Similar effects were recorded by Carpanini *et al.* (1978) when 800 ppm allyl alcohol was given in the drinking-water to rats, but again there was no evidence of local irritation even though allyl alcohol is known to be irritant to mucous membranes (Dunlap, Kodama, Wellington, Anderson & Hine, 1958). It may be that the increase in weight was due, at least in part, to an increase in enzyme activity, in view of the evidence that this ester is hydrolysed rapidly in the intestine (Longland *et al.* 1978).

The relative weight of the kidneys was increased at wk 14 in animals given the highest dose level, but no significant histopathological changes were detected and there were no changes in the urinary cell excretion. The increases were statistically significant, however, and it is likely that the heavier kidneys found at wk 14 represent a true effect of allyl caproate. Similar effects on the kidneys were found at autopsy when allyl alcohol was administered to rats (Carpanini *et al.* 1978; Dunlap & Hine, 1955; Dunlap *et al.* 1958; Torkelson, Wolf, Oyen & Rowe, 1959). However, Carpanini *et al.* (1978) considered that the kidney effects at low dose levels were unusual, since the liver is the generally accepted target organ of allyl alcohol.

Significant increases in the relative weights of the spleen compared to those of the controls were seen at each examination in all groups of animals given 100 mg allyl caproate/kg, except females at wk 2 and males at wk 6. Although there were no marked pathological changes in the spleen and no significant damage to the spleen has been reported in other

studies of allyl caproate and allyl alcohol, the increases found in the present study must, in the absence of evidence to the contrary, be attributed to allyl caproate.

The increases in liver weight or relative liver weight at all the time intervals in rats given the highest dose level, and at wk 14 in those given 35 mg allyl caproate/kg, must be regarded as being due to treatment. This assumption is confirmed by the histopathological changes seen in this organ. Periportal vacuolation was found in a high percentage of the livers of the animals examined and the incidence of this condition was dose related. The finding of periportal necrosis in rats given 100 mg allyl caproate/kg confirms the findings of the short-term study of Hagan *et al.* (1967). Reid (1972) found that ip doses of 50 mg allyl alcohol/kg had hepatotoxic effects similar to those found in this study, as did Rees & Tarlow (1967) on giving oral doses of either allyl alcohol or allyl formate. The finding that allyl caproate given by oral intubation produces an incidence of liver necrosis similar to that produced by allyl alcohol administered by this method is in keeping with the evidence that allyl caproate is hydrolysed rapidly in the small intestine to allyl alcohol (Longland *et al.* 1977). Support is found also for the hypothesis of Carpanini *et al.* (1978) that the mode of administration plays a significant part in the outcome of allyl alcohol (and allyl ester) treatment. Liver necrosis was found in the present study when the ester was given orally by intubation, but Carpanini *et al.* (1978) found no such effect when allyl alcohol was given in the drinking-water.

Since these investigations detected pronounced effects on the livers of rats given 12 mg allyl caproate/kg/day or more, it was not possible to determine a no-toward-effect level for this ester.

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SHORT-TERM TOXICITY OF *n*-AMYL ALCOHOL IN RATS

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Abstract—*n*-Amyl alcohol, dissolved in corn oil, was administered to rats by oral intubation at dose levels of 0 (control), 50, 150 or 1000 mg/kg body weight/day for 13 wk. The treatment had no demonstrable effect on body-weight gain, food and water consumption, haematological values, serum and urine analyses, renal function, organ weights or histopathology. The no-untoward-effect level was 1000 mg/kg/day, which is about 2000 times the estimated maximum likely intake by man.

INTRODUCTION

n-Amyl alcohol (pentyl alcohol; 1-pentanol; *n*-butylcarbinol) is currently used in the UK and Europe as a constituent of flavouring agents. In a recent report of the Food Additives and Contaminants Committee (1976) it is classified on available evidence as a flavouring acceptable for use in food, subject to a limit, on technological grounds of 50 ppm in the diet. However, it is stated that the toxicologically acceptable level could be higher. The Council of Europe (1974) classified *n*-amyl alcohol as a permissible food additive with an Acceptable Daily Intake of 1 mg/kg body weight. In the USA, it is permitted for use as a synthetic flavouring substance and additive under Sec. 121.1164 of the Code of Federal Regulations. An unpublished survey carried out by BIBRA in 1968 revealed maximum daily ingestion levels in man of 33 mg in the UK and 42 mg in the USA and Europe.

Haggard, Miller & Greenberg (1945) reported that *n*-amyl alcohol was oxidized to valeric acid by way of the aldehyde in rats, and according to Gaillard & Derache (1964) less than 0·03% of a single oral dose was excreted in the urine of rats within 8 hr. On the basis of the quantity required to cause respiratory failure, Haggard *et al.* (1945) found *n*-amyl alcohol to be 12 times more toxic than ethanol following ip administration. By the oral route, however, the difference in toxicity would not be expected to be as great, since the maximun blood concentration reached by *n*-amyl alcohol was one-seventh of that reached by ethanol (Gaillard & Derache, 1964). The oral LD_{50} in rats reported by Jenner, Hagan, Taylor, Cook & Fitzhugh (1964) was 3·03 g/kg, whereas that for ethanol was reported by Smyth, Seaton & Fischer (1941) to be 13·7 g/kg. The only observed effect of an acute oral dose of *n*-amyl alcohol was central nervous system depression. Published data on the effects of prolonged administration of this alcohol are restricted to the observations of Straus & Blocq (1887), who reported liver necrosis in the rabbit following repeated oral administration for up to 1 yr. Damage to the mucosa of the stomach was the only other pathological finding reported. Acute poisoning in man, as reviewed by Treon (1963), includes irritation of the eyes and respiratory tract, vertigo, dyspnoea, cough, nausea, vomiting and diarrhoea. Glycosuria and methaemoglobinemia also have been reported.

There is a similar lack of information on isoamyl alcohol. No data are available on prolonged administration to animals, but in a short-term study in rats, Carpanini, Gaunt, Kiss, Grasso & Gangolli (1973) found only that there was a slightly reduced rate of body-weight gain in the males given the highest dose level (1000 mg/kg/day) as a result of a reduction in food intake.

In the absence of more recent data on the effects of prolonged administration of *n*-amyl alcohol, the present study has been performed as part of a safety evaluation programme for flavouring agents.

EXPERIMENTAL

Materials. *n*-Amyl alcohol was supplied by NV Chemische Fabriek Naarden, The Netherlands, and complied with the following specification: A clear, colourless liquid; purity, min. 97%; specific gravity (20°/20°C), 0·815–0·816; refractive index (20°C), 1·410; m.p., −78·9°C; b.p., 137·8°C; arsenic, max 3 ppm; copper, max 50 ppm; iron, max 50 ppm; lead, max 10 ppm; total heavy metals (as lead), max 20 ppm.

Animals and diet. Rats of the ASH/CSE strain obtained from a specified-pathogen-free breeding colony were fed Spillers' Laboratory Small Animal Diet and given tap-water *ad lib.* The animal rooms were maintained at 20 ± 1°C with a relative humidity of 40–60%.

Experimental design and conduct. *n*-Amyl alcohol was given by daily oral intubation (7 days/wk) to groups of 15 male and 15 female rats for 13 wk at dose levels of 0 (control), 50, 150 and 1000 mg/kg body weight/day. In addition, groups of five rats of

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Table 1. *Mean body weights and food and water consumption values of rats given daily doses of 0–1000 mg* n-*amyl alcohol/kg/day for 13 wk*

Dose level (mg/kg/day)	Body weight (g) at day 0*	34	62	91	Mean food consumption (g/rat/day)	Mean water consumption (ml/rat/day)
			Males			
0	93	323	424	467	18·8	26·2
50	91	326	422	470	18·9	24·3
150	91	336	449	504	19·6	24·3
1000	96	316	426	479	18·0	24·3
			Females			
0	91	211	256	281	14·7	20·6
50	91	211	259	286	14·9	20·5
150	89	217	268	293	15·1	21·6
1000	92	205	250	276	14·9	22·1

*Value on first day of dosing.
Values of body weights are means for 15 animals. Values of food and water consumption are calculated from weekly means for three cages of five animals.
No values differed significantly from those of controls as $P < 0{\cdot}05$ using Student's t test for body weights, and the ranking test of White (1972) for overall consumptions.

each sex, with body weights similar to those of the four main groups, were given daily doses of 0, 150 or 1000 mg *n*-amyl alcohol/kg/day and were killed after 2 or 6 wk. The *n*-amyl alcohol was dissolved in corn oil in appropriate concentrations, so that all rats received a dosage volume of 5 ml/kg/day. The animals were weighed initially, at days 1, 2 and 6 and then at intervals of not more than 1 wk up to day 91 of the study. Food and water consumptions were measured over the 24-hr period preceding the day of weighing.

After the final dose the animals were deprived of food for 24 hr and killed by exsanguination from the abdominal aorta under barbiturate anaesthesia, the blood being retained for haematological examination and serum analyses. The blood was examined for haemoglobin content, packed cell volume and counts of erythrocytes and leucocytes. Slides were prepared from all blood samples for the counting of reticulocytes and the different types of leucocytes, but the counts were confined to the controls and the animals on the highest level of treatment except at wk 2, when leucocyte counts were also made on the 150-mg/kg groups. At wk 13, serum was analysed for urea, glucose, total protein and albumin as well as for the activities of glutamic–oxalacetic transaminase, glutamic–pyruvic transaminase and lactic dehydrogenase.

At autopsy all the tissues were examined for gross abnormalities and the brain, heart, liver, spleen, kidneys, stomach, small intestine, caecum, adrenals, gonads, pituitary and thyroid were weighed. Samples of these organs and of lung, lymph nodes, salivary gland, trachea, oesophagus, aortic arch, thymus, urinary bladder, colon, rectum, pancreas, uterus and skeletal muscle were preserved in 10% buffered formalin. Paraffin-wax sections of these tissues were stained with haematoxylin and eosin for microscopic examination, which was carried out on liver and kidney sections from all animals but on other types of tissue from only half of the control rats and from those given 1000 mg *n*-amyl alcohol/kg for 13 wk.

During wk 2 and 6 urine was collected over a 6-hr period of water deprivation from the rats about to be killed. Similar samples were collected from the remaining rats during wk 12. The volumes and specific gravities of these samples were measured to determine the renal concentrating ability of the rats. In addition, the samples were examined for their appearance, number of cells and content of albumin, glucose, ketones, bile salts and blood. At wk 6 and 12 the concentration test was extended to include measurements of the volume and specific gravity of urine produced in a 4-hr period commencing after 16 hr without water. In addition, the renal diluting capacity was investigated by measuring the volume and specific gravity of urine produced in the first 2 hr after a water load of 25 ml/kg.

RESULTS

No abnormalities in appearance or behaviour were seen during the study and there were no significant differences between the treated and control rats in body weight or in food and water consumption (Table 1).

Only isolated differences from the controls were seen in the results of the haematological studies (Table 2). These included a lower total leucocyte count at wk 2 in the male rats given 150 or 1000 mg *n*-amyl alcohol/kg/day and lower haemoglobin concentrations at wk 13 in the male animals given 50 or 1000 mg/kg/day. Also there were higher percentages of reticulocytes in the male rats given 1000 mg/kg/day at wk 2 and in the females at wk 13, as well as a slightly lower percentage of lymphocytes at wk 6 in the females given 1000 mg/kg/day. The results of the serum analyses were similar in test and control rats.

The urine was free from bile, blood, glucose and ketones, while the concentration of albumin was similar in all groups. At wk 6 there were lower cell counts in the urine of the male rats given 150 or 1000 mg *n*-amyl alcohol/kg/day, the differences being statistically significant ($P < 0{\cdot}01$). Some statistically signifi-

Table 2. *Haematological values (aortic blood) for rats given daily doses of 0–1000 mg* n-*amyl alcohol/kg/day*

Sex and dose level (mg/kg/day)	No. of rats	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 2										
Male										
0	5	12·7	41	5·40	1·4	7·1	11	1	85	3
150	5	12·7	41	5·80	—	5·5*	11	0	86	3
1000	5	13·0	42	5·15†	2·3***	5·0**	12	1	85	2
Female										
0	4	13·5	45	5·93	1·3	3·7	11	0	87	2
150	5	12·6	44	5·85	—	4·5	10	0	89	1
1000	5	13·0	44	5·74	1·0	4·6	11	1	86	2
Wk 6										
Male										
0	5	14·5	47	6·79	0·5	8·3	11	0	88	1
150	5	14·6	48	6·97†	—	8·9†	—	—	—	—
1000	5	14·0	46	6·76	0·4	5·6†	7	0	91	2
Female										
0	5	14·3	45	6·70	0·2	5·6	8	0	91	1
150	5	13·7	43	6·60	—	5·5	—	—	—	—
1000	5	14·1	44	6·57	0·1	6·6	18	1	79*	2
Wk 13										
Male										
0	14	14·1	45	6·90	0·6	5·6	17	1	80	2
50	15	13·4***	43	6·58	—	5·4	—	—	—	—
150	15	13·9	44	6·64	—	5·4	—	—	—	—
1000	15	13·6**	44	6·66	0·8	5.4	18	2	79	2
Female										
0	13	14·0	43	6·56	0·3	4·7	10	1	87	2
50	14	13·5	43	6·51	—	4·4	—	—	—	—
150	14	13·8†	43†	6·45	—	4·4	—	—	—	—
1000	15	13·7	43†	6·65†	0·6**	3·9	12	1	85	2

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

†No. of rats one less than indicated.

Values are means for the numbers of rats shown, and those marked with asterisks differ significantly (Student's *t* test) from the control value: $*P<0·05$; $**P<0·01$; $***P<0·001$.

Basophils did not account for more than 0·5% of the leucocytes in any group and inclusions were not seen in the erythrocytes.

cant ($P<0·05$) differences were also apparent in the concentration tests at wk 12; the specific gravity of the samples collected at 16–20 hr from females given 1000 mg/kg/day was higher than the control value and the volume was lower. After the same period on test, the male rats given 50 or 1000 mg/kg/day produced less urine in the 6-hr period without water. No differences from the controls were found in the dilution tests or at wk 2 and 6 in the concentration tests.

Examination of the organ weights showed some isolated differences at wk 2, but none thereafter. The stomach weights in the males and females given 1000 mg *n*-amyl alcohol/kg/day were higher than those of the controls (Table 3), but the difference was confined to the male rats when the values were related to body weight. Also, a higher heart weight was found in the female rats given the top level of treatment, but this was not evident when the figure was related to body weight. Relative to body weight, the spleens from the female rats dosed with 1000 mg/kg/day showed a low value, as did the female kidney weights, both at this and at the 150-mg/kg/day level.

At autopsy, no abnormalities were seen at any dose level. On histological examination, protein casts and foci of calcification were found in the kidney tubules, particularly from the male animals, but the incidences were similar in the treated animals and their corresponding controls. The incidence of fatty change and inflammatory cell infiltration in the liver was again comparable in the control and treated rats. No histological changes related to the period or level of treatment were seen in any of the organs examined.

DISCUSSION

Few effects were found in this study and the animals appeared healthy throughout. Only isolated changes were found in the haematological studies. There was no consistent pattern in any of these findings either with respect to dose-response, sex or time relationships. The reductions in haemoglobin concentration in the male rats at wk 13 suggest a mild anaemia, but this is not supported by other measurements. The packed cell volumes, mean corpuscular haemoglobin concentrations and erythrocyte and reti-

Table 3. *Mean relative organ weights of rats given daily doses of 0–1000 mg n-amyl alcohol/kg/day for 2 or 13 wk*

Sex and dose level (mg/kg/day)	No. of rats	Relative organ weight (g/100 g body weight)												Terminal body weight (g)
		Brain	Heart	Liver	Spleen	Kidneys	Stomach	Small intestine	Caecum	Adrenals†	Gonads‡	Pituitary†	Thyroid†	
							Wk 2							
Male														
0	5	1·04	0·45	3·59	0·36	0·88	0·62	4·75	0·47	24·24	1·25	3·5	8·0	164
150	5	0·99	0.47	3·68	0·38	0·90	0·70	4.89	0·49	25·42	1·21	3·5	6·9	164
1000	5	0·98	0·46	3·75	0·37	0·92	0·72*	4·47	0·49	27·87	1·28	4·0	6·7	164
Female														
0	5	1·14	0·44	4·13	0·38	0·96	0·70	4·45	0·42	35·36	61·7	6·3	8·4	137
150	5	1·11	0·46	3·83	0·33	0·86*	0·64	4·14	0·43	35·84	60·9	6·1	9·2	144
1000	5	1·05	0·47	3·74	0·31*	0·85**	0·68	3·84	0·44	36·71	54·8	5·9	9·2	150
							Wk 13							
Male														
0	15	0·42	0·31	2·68	0·17	0·64	0·41	2·02	0·22	15·66	0·83	2·9	4·7	456
50	15	0·42	0·30	2·73	0·18	0·63	0·42	2·19	0·21	15·05	0·81	2·6	4·8	461
150	15	0·40	0·30	2·72	0·19	0·61	0·41	2·21	0·23	14·76	0·80	2·7	4·4	491
1000	15	0·41	0·31	2·69	0·19	0·64	0·43	1·98	0·23	15·19	0·81	2·8	4·9	474
Female														
0	15	0·66	0·34	2·49	0·25	0·63	0·53	2·52	0·29	28·10	54·9	5·2	7·0	270
50	15	0·66	0·34	2·49	0·24	0·65	0·53	2·53	0·31	29·45	53·2	5·3	6·5	268
150	15	0·63	0·35	2·49	0·25	0·64	0·53	2·59	0·29	27·74	54·7	5·1	6·6	276
1000	14	0·66	0·37	2·36	0·24	0·62	0·50	2·43	0·31	28·36	54·0	4·7	7·5	268

†Weights of this organ are expressed in mg/100 g body weight.
‡Weights of the female gonads are expressed in mg/100 g body weight.
Values are means for the numbers of animals shown and those marked with asterisks differ significantly (Student's *t* test) from the control value: $*P<0.05$; $**P<0.01$.

culocyte counts were all within normal limits (Hardy, 1967) and there were no variations in spleen weight. Although at wk 2 the male animals given 150 or 1000 mg/kg/day had low total white cell counts, these were probably chance findings since the corresponding female rats did not show a similar effect and the differential white cell counts were very similar to those of the controls. Although there were some variations in the reticulocyte counts, all these were within the normal range for rats of this strain (Hardy, 1967).

No specific gravity or volume measurements of the urine indicated any adverse effect on renal function in the treated groups. The two isolated reductions in relative kidney weights were not associated with any histopathological findings, nor were similar changes evident after more prolonged treatment. These considerations, together with a lack of effect on the absolute kidney weight, cannot be taken to indicate an effect of treatment.

As *n*-amyl alcohol was administered by gastric intubation, it is probable that the increased stomach weights found at wk 2 in some rats given the top dose were associated with a mild irritation due to the mode of administration. There was no histological evidence of such an irritation at any stage of this study, but such effects have been reported in rabbits (Straus & Blocq, 1887), and irritation of the mucosa is known to occur in cases of acute poisoning with this alcohol in man (Treon, 1963). The effect did not persist throughout the study. The few other changes in organ weights were isolated, followed no consistent pattern and were considered to be unrelated to treatment.

In their short-term study on isoamyl alcohol administered by gastric intubation to rats, Carpanini *et al.* (1973) found a similar lack of harmful effects. In that investigation, the no-untoward-effect level was 1000 mg/kg/day, which was 350–400 times the calculated maximum daily intake by man.

From the results of this study, it is concluded that the no-untoward-effect level for *n*-amyl alcohol in the rat is, like that of isoamyl alcohol, at least 1000 mg/kg/day, which is about 2000 times the estimated maximum likely intake by man. In this study the application of, for example, a 100-fold safety factor suggests an acceptable level of intake for man of 10 mg/kg/day. The Acceptable Daily Intake deduced from currently available data by the Council of Europe (1974) was only 1 mg/kg.

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INHIBITION OF NITROSAMINE FORMATION *IN VITRO* BY SORBIC ACID

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Abstract—Sorbic acid (*trans,trans*-2,4-hexadienoic acid), a food preservative, was found to react rapidly with nitrite in acidic media. One of the products of the reaction was isolated and identified as an oxime nitrite of sorbic acid. Sorbic acid inhibited the *in vitro* formation of *N*-nitrosodimethylamine from dimethylamine and nitrite. The extent of the inhibition by sorbic acid was approximately the same as that by ascorbic acid under comparable conditions. Against the formation of *N*-nitrosomorpholine, the inhibitory action of sorbic acid was weaker than that of ascorbic acid, and unlike the latter acid, sorbic acid had no inhibitory effect on the nitrosation of *N*-methylaniline.

INTRODUCTION

Several agents are known to inhibit the formation of nitrosamines from amines and nitrite (Mirvish, 1975; Mirvish, Wallcave, Eagan & Shubik, 1972). The most prominent among these is ascorbic acid, since it is highly effective, is relatively non-toxic itself, and therefore may be added to foods. This inhibition results from a rapid interaction between ascorbic acid and nitrite, the kinetic details of which have already been documented (Dahn, Loewe & Bunton, 1960). We have found that sorbic acid (*trans,trans*-2,4-hexadienoic acid; $CH_3 \cdot CH{:}CH \cdot CH{:}CH \cdot COOH$), a food preservative, reacts rapidly with nitrite under acidic conditions. This paper describes the characteristics of the reaction and the inhibitory action of sorbic acid on *in vitro* nitrosamine formation from amines and nitrite.

EXPERIMENTAL

Materials. The sorbic acid, ascorbic acid and sodium nitrite used were reagent-grade commercial samples supplied by Wako Pure Chemical Industries, Osaka. *N*-Nitrosodimethylamine and *N*-nitroso-*N*-methylaniline were also commercial products supplied by Wako Pure Chemical Industries and Kokusan Chemical Works, Tokyo, respectively. *N*-Nitrosomorpholine was prepared by nitrosation of morpholine with nitrous acid. Thin-layer chromatography was carried out on silica gel 60 F254, Merck AG (Darmstadt, Germany), using the following solvent systems: (1) *n*-hexane–dioxane–acetic acid (9:5:1, by vol.); (2) chloroform–methanol (95:5, v/v); (3) chloroform–isobutyric acid (9:1, v/v); (4) isobutyric acid–0·5 N-ammonia (10:6, v/v).

Kinetic studies of the reactions of sorbic and ascorbic acids with nitrite. A solution (10 ml) of sorbic acid (1 mM) in a buffer of pH 1, 2, 3, 4 or 5 was maintained at 37°C. The buffers used were 0·05 M-sodium citrate adjusted to the required pH by addition of 60% perchloric acid. To the sorbic acid solution was then added 0·1 ml 10 mM-sodium nitrite, and the mixture was allowed to stand at 37°C. Portions of 0·1 ml were removed from the solution, mixed with 5 ml water and 0·4 ml Griess reagent (sulphanylic acid and 1-naphthylamine in 20% acetic acid), and the resulting mixtures were left at 37°C for 15 min. Absorbances at 520 nm were a measure of the nitrite content of these solutions. The pH of the reaction mixture was checked both at the start and finish of incubation and was generally unchanged.

The reactions between ascorbic acid and nitrite were carried out in the same way, apart from a modification of the nitrite-determination process. The portions (0·1 ml) removed from the reaction mixture were first incubated in 0·1 M-NaOH (5 ml) at 37°C for 10 min to destroy the ascorbic acid, and then treated with Griess reagent (0·4 ml, containing 75% acetic acid) to develop the colouration. This modification was made because treatment with Griess reagent without prior incubation in the alkali gave very low nitrite values; apparently the ascorbate continued to react with the nitrite during the colour-developing process.

Product analysis by thin-layer chromatography. Reaction mixtures consisting of various combinations of sorbic acid and nitrite concentrations were incubated for times ranging from 0·5 to 5 hr and extracted exhaustively with ether. The ether solutions were concentrated and subjected to chromatography using solvent 1. The spots on the chromatograms were visible under ultraviolet light of 254 and 365 nm. The reaction mixtures used are given in Table 1.

Preparation of the oxime nitrite of sorbic acid. Three different methods of preparing the oxime nitrite (compound I in Fig. 1) on a larger scale were attempted.

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Table 1. *Reaction conditions resulting in the thin-layer chromatograms shown in Fig. 3*

Parameter	TLC Lane no.* (See Fig. 3)								
	I	II	III	IV	V	VI	VII	VIII	IX
pH	1	3	4	1	1	1	1	**	**
Time (hr)	0·5	1	5	3	3	3	3	3	3
Sorbic acid (mM)	1	1	1	1	1	10	10	190	190
Nitrite (mM)	0·1	0·1	0·1	1	10	1	100	380	1900

*The incubation temperature was 37°C for the reactions of lanes I–VII and 0°C for those of lanes VIII and IX.

**Conc. HCl–acetic acid–water, 12:20:20, v/v.

Method (a). Nitrogen trioxide (N_2O_3) was generated by addition of an aqueous solution of sodium nitrite (70 g in 160 ml water) into concentrated sulphuric acid (54 ml), the addition taking a period of 2 hr, during which the N_2O_3 generated was continuously introduced into an ice-cooled solution of sorbic acid (11·2 g) in chloroform (350 ml). The precipitate was collected by filtration and washed with chloroform giving a slightly coloured powder weighing 9·7 g (52%) after drying. This powder was recrystallized five times from hot water to give platelets that melted at 165–167°C. Analysis found: C, 38·29; H, 4·08; N, 14·99; $C_6H_8N_2O_5$ requires C, 38·30; H, 4·28; N, 14·89; O, 43·53%; Mass: *m/e*, 188. Ultraviolet absorption: at pH 1·0, λ_{max} 252 nm (ϵ, 15,600); at pH 7·0 λ_{max} 235 nm (ϵ, 21,100); at pH 12·0 λ_{max} 223 nm (ϵ, 14,600), λ_{max} 290 nm (ϵ, 21,750), λ_{min} 252 nm (ϵ, 7900). Infrared spectrum (KBr): –OH, 2800–3600 cm^{-1}; C=O, 1670 cm^{-1}; C=C, 1560 cm^{-1}; C=N and N=O, 1600–1650 cm^{-1}. N.m.r. in [^{2}H]dimethylsulphoxide : δ (ppm), tetramethylsilane as the internal standard; CH_3 at position 6, 1·68 (d, J = 8 cps); CH at position 5, 5·94 (q, J = 8 cps); CH at position 3, 6·31 (d, J = 16 cps); CH at position 2, 7·10 (d, J = 16 cps).

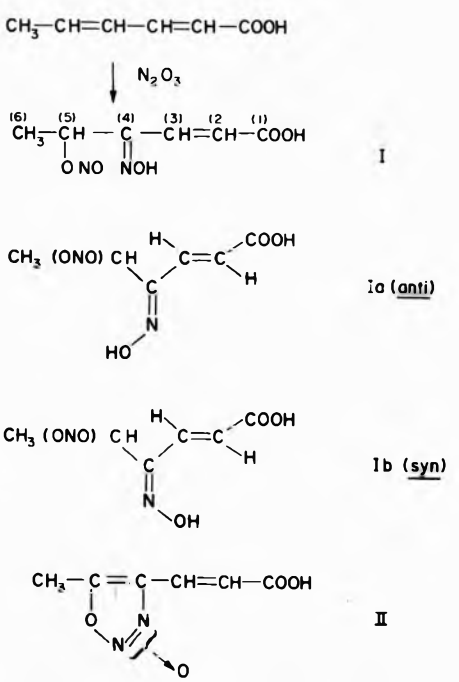

Fig. 1. Structures of the products of the sorbic acid–nitrite reaction.

When the crude precipitate was recrystallized only twice from water, the material obtained (m.p. 148–150°) was a mixture of the *anti* and *syn* conformers of the oxime nitrite (compounds Ia and Ib in Fig. 1). This was found from the n.m.r. spectrum of the compound in [^{2}H]dimethylsulphoxide. The minor component Ib (about one third of Ia) gave the following signals: δ (ppm), CH_3, *c.* 1·6; CH at position 5, c. 6.0; CH at position 3, *c.* 6·3; CH at position 2, 7·6. This material gave single spots on thin-layer chromatographs run in solvents 1–4. Elemental analysis of the material gave values consistent with the oxime nitrite structure: C, 38·40; H, 4·26; N, 14·70%. Both infrared and ultraviolet spectra were identical with the corresponding spectrum of pure compound Ia (Fig. 1).

Method (b). Sorbic acid (11·2 g) was suspended in a mixture of concentrated hydrochloric acid (120 ml), acetic acid (200 ml) and water (200 ml). Sodium nitrite (20·7 g) was added slowly over a period of 1 hr to the suspension, which was cooled in an ice-bath and mechanically stirred. During this treatment, the sorbic acid went into solution. The red solution which resulted after a further 2 hr in the ice-bath was extracted three times with ether (500 ml each). The ether layer was collected and evaporated to dryness under reduced pressure. The residue was dissolved in ether (500 ml) and this solution was washed with water (500 ml × 3) and dried with sodium sulphate. On evaporation of the ether, a yellow oil (13.2 g) was obtained, which was suspended in chloroform (20 ml) and the suspension was allowed to stand in a refrigerator for 2 days. The slightly coloured precipitate of the oxime nitrite, thus obtained, was collected by filtration (yield, 5·9 g; 31%). This material was equivalent to the crude substance obtained by the first method described, consisting of Ia and Ib (Fig. 1).

Method (c). To 500 ml of 10 mM-sorbic acid in 0·1 M-HCl–0·05 M-KCl, pH 1·0, was added 50 mmol sodium nitrite. The mixture was adjusted to pH 1 by adding conc. HCl and was allowed to stand at 37°C for 3 hr.

Ether (200 ml × 3) was used to extract the product; the ether solution was washed with water (600 ml) and dried with sodium sulphate. Evaporation of the ether gave an oil (0·46 g) which was submitted to preparative thin-layer chromatography using solvent 2. The band corresponding to the oxime nitrite I (R_F, 0·2) was eluted with ether

to give 0·07 g of a powder. This powder was of the same quality as the crude oxime nitrite described in methods (a) and (b), as judged by its n.m.r. spectrum and thin-layer chromatogram.

Another major product was obtained as a yellow oil (0·05 g) from the band at R_F 0·4 by elution with ether. This material showed λ_{max} 229 nm at pH 1, 278 nm at pH 7 and 282 nm at pH 12·6. When subjected to thin-layer chromatography in solvent 1, this oily material showed one major and three minor bands. Although further purification by repeated preparative thin-layer chromatography was carried out, a pure compound was unobtainable.

Isomerization of oxime nitrite (compound Ia—Fig. 1) by alkali. To a suspension of pure Ia (50 mg) in 1 ml water, was added 0·35 ml 1 M-NaOH. The resulting yellow solution, at pH 12·1, was allowed to stand at 20°C for 1 hr. The solution was then adjusted to pH 3 with 0·02 ml conc. HCl and a small amount of 1 M-HCl. The product was extracted with ether (2 ml × 5) and the ether solution was dried with sodium sulphate. On evaporation of the ether, a colourless powder (33 mg) was obtained. On thin-layer chromatographic analysis, this powder was found to contain the dehydrated oxime nitrite (compound II—Fig. 1) in addition to oxime nitrite I. The n.m.r. spectrum of this material in [^{2}H]dimethylsulphoxide revealed that it consisted of compounds Ia, Ib and II (Fig. 1) in an approximate molar ratio of 2:1:1.

Dehydration of oxime nitrite I. Oxime nitrite I (recrystallized once from water; 188 mg) was dissolved in 0·1 M-HCl–0·05 M-KCl (pH 1·0; 100 ml) and the solution was incubated at 37°C for 48 hr. The solution was extracted with ether (100 ml × 3) and the ether layer was washed with water (300 ml) and dried with sodium sulphate. The ether was evaporated to dryness to give a powder of the dehydrated product (89 mg) which was recrystallized from hot water to give a pure sample of compound II (19 mg) with m.p. 160–163°C. Analysis found: C, 42·20; H, 3·49; N, 16·31%; $C_6H_6N_2O_4$ requires C, 42·36; H, 3·56; N, 16·47. Mass *m/e*, 170. Ultraviolet absorption: at pH 1·0, λ_{max} 232 nm (ε, 24,000); at pH 7·0, λ_{max} 226 (ε, 24,300), shoulder at 270 nm; at pH 12·0, λ_{max} 226·5 nm (ε, 24,650), shoulder at 270 nm. N.m.r. in [^{2}H]dimethylsulphoxide with tetramethylsilane as internal standard: δ (ppm): CH_3 at position 6, 2·30 (s); CH at position 3, 6·67 (d, J = 16 cps); CH at position 2, 7·40 (d, J = 16 cps).

To measure the velocity of the dehydration, compound I (2 mg) was incubated in 0·1 M-HCl–0·05 M-KCl (1 ml), pH 1·0, at 37°C. Samples (50 μl) of the solution were diluted with 5 ml 0·1 M-NaOH–0·1 M-Na_2HPO_4 at pH 12, and the absorbances at 288 nm were recorded. From the decrease in the absorbance, the extent of the dehydration was determined.

Inhibition of in vitro *N-nitrosodimethylamine formation.* Dimethylamine was incubated with sodium nitrite in the presence or absence of sorbic acid. The medium used was 0·05 M-sodium citrate adjusted to pH 2, 3 or 4 with perchloric acid. Concentrations of the reagents are specified in Table 2. The reaction mixture (15 ml) was kept in a stoppered vessel at 37°C for 2, 5 or 8 hr. The pH of the mixture was frequently measured and adjusted when necessary. At the end of the incubation, the pH of the mixture was raised to 13 with sodium hydroxide to stop the reaction. After addition of sodium chloride (5 g), the mixture was extracted with methylene chloride (20 ml × 2) and the solution was dried with sodium sulphate. The solution was made up to 50 ml by addition of more methylene chloride. (This extraction procedure was found to be 96% efficient in a separate experiment.) To 1 ml of the solution, 10 μl of 0·10 M-*n*-heptanol in chloroform was added as the reference, and the resulting mixture (1–1·5 μl) was subjected to gas chromatography. The chromatography was carried out on a Shimadzu Gas Chromatographic Analyzer GC-4B equipped with a flame ionization detector. The columns (4 × 100 mm; temperature 70°C) were filled with 5% PEG 20 M (Chromosorb-W, 60–80 mesh),

Table 2. *Inhibition of dimethylamine nitrosation by sorbic acid**

pH	$NaNO_2$ (mM)	Sorbic acid (mM)	Ascorbic acid (mM)	Time (hr)	Dimethylnitrosamine formed (mM)		Inhibition (%)	
					Exp. 1	Exp. 2	Exp. 1	Exp. 2
2·0	40	—	—	8	1·35	1·33	0	0
	40	20	—	8	0·48	0·35	64	74
	40	—	20	8	0·47	0·54	65	59
3·0	20	—	—	5	4·2	—	0	—
	20	20	—	5	1·4	—	67	—
	20	—	20	5	0·6	—	86	—
	40	—	—	8	10·40	11·60	0	0
	40	20	—	8	4·90	5·12	53	56
	40	—	20	8	4·60	4·68	56	60
	80	—	—	2	20·0	—	0	—
	80	20	—	2	11·6	—	42	—
	80	—	20	2	11·7	—	41	—
4·0	40	—	—	8	10·50	12·50	0	0
	40	20	—	8	6·20	7·19	41	42
	40	—	20	8	7·00	8·47	33	32

*The concentration of dimethylamine was 500 mM and the incubation temperature was 37°C.

and the carrier gas was nitrogen. The amount of the nitrosamine was determined by comparing the peak height of the nitrosamine with that of the internal reference.

Inhibition of the nitrosamine formation by ascorbic acid was investigated in the same way.

Inhibition of the formation of N-*nitrosomorpholine and* N-*nitroso*-N-*methylaniline.* These inhibitions were examined in a manner similar to that described for the inhibition of *N*-nitrosodimethylamine formation. The concentrations of the reagents and the incubation periods are specified in Table 3. The reference compounds in the gas-chromatographic analysis were naphthalene for nitrosomorpholine and acenaphthene for nitrosomethylaniline.

Examination of genetic toxicity. Salmonella typhimurium, strains TA98, TA100 (McCann, Spingarn, Kobori & Ames, 1975) and TA1538 (Ames, Lee & Durston, 1973) were used to test the mutagenicity of compound I and the oily unidentified product x in Fig. 3, both in the presence and absence of rat liver S-9 fraction (Ames, Durston, Yamasaki & Lee, 1973). The levels of test compounds ranged from 10 to 100 μg/8-cm round-plate.

To test the mutagenicity of the reaction mixture itself, an aqueous solution of pH 1 containing 10 mM-sorbic acid and 100 mM-sodium nitrite was incubated at 37°C. At intervals, 5-ml portions were mixed with an excess of sulphamic acid (50 mg) to degrade nitrite, and neutralized with a small amount of sodium hydroxide; 0·1 ml of this solution was assayed.

The DNA-damaging activity of compounds was measured using *Bacillus subtilis* H17 and M45 strains by a method already described (Kada, Tutikawa & Sadaie, 1972).

RESULTS

Nature of the reaction between sorbic acid and nitrite

The decreases in nitrite concentration when it was mixed with sorbic acid in aqueous solutions were measured. Figure 2 shows the time courses of the losses at various pH values of 37°C, in mixtures of 0·1 mM-nitrite and 1 mM-sorbic acid. It can be seen that the losses were rapid at pH 1 to pH 3, considerably slower at pH 4 and very slow at pH 5. In the control experiment, in which the nitrite was incubated at the desired pH value in the absence of sorbic acid, the nitrite loss was insignificant (Fig. 2). The reactions

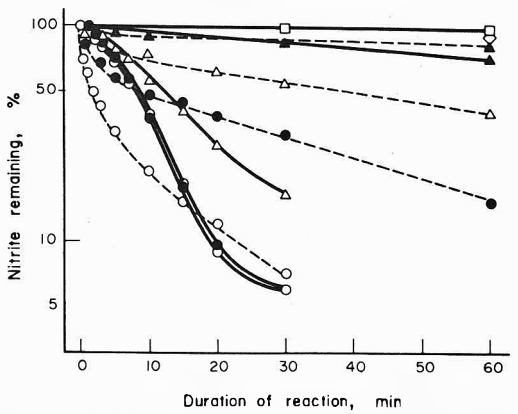

Fig. 2. Time-course of the reaction of 0·1 mM-sodium nitrite with 1 mM-sorbic acid (——) or 1 mM-ascorbic acid(– – –) at 37 C and pH 1 (○), 2 (●), 3 (△), 4 (▲) and 5 (□). The control experiment, in which 0·1 mM-sodium nitrite was incubated at pH 1 without addition of sorbic acid, is represented by ◇.

Table 3. *Inhibition of the nitrosation of morpholine and* N-*methylaniline by sorbic acid*

pH	Sorbic acid (mM)	Ascorbic acid (mM)	Nitrosamine formed (mM) Exp. 1	Nitrosamine formed (mM) Exp. 2	Inhibition (%) Exp. 1	Inhibition (%) Exp. 2
			Morpholine*			
2·0	—	—	2·08	1·95	0	0
	20	—	0·75	0·54	64	72
	—	20	0·11	0	95	100
3·0	—	—	5·00	4·29	0	0
	20	—	2·90	2·28	42	47
	—	20	0·37	0·18	93	96
4·0	—	—	2·58	2·51	0	0
	20	—	2·17	1·70	16	32
	—	20	0·57	0·44	78	83
			***N*-Methylaniline†**			
2·0	—	—	5·82	6·17	0	0
	20	—	5·86	6·09	0	1
	—	20	1·48	1·37	75	78
3·0	—	—	5·78	6·13	0	0
	20	—	5·90	6·06	0	1
	—	20	1·80	1·76	69	71
4·0	—	—	5·52	5·39	0	0
	20	—	6·08	6·13	0	0
	—	20	2·40	2·03	56	62

*Morpholine (20 mM) was incubated with nitrite (20 mM) at 37°C for 1 hr.
†*N*-Methylaniline (10 mM) was incubated with nitrite (10 mM) at 37°C for 10 min.

at pH 1 and pH 2 proceeded at almost identical rates. The sigmoidal shape of the curves was reproducible in repeated experiments and may reflect the complex nature of this reaction. A similar curve shape was seen at pH 3. At all the pH values examined, the initial rates were higher for the ascorbic acid–nitrite interactions than for the sorbic acid–nitrite interactions, but as the reactions progressed this situation was reversed.

Analysis of the reaction mixtures by thin-layer chromatography on silica gel indicated that the sorbic acid–nitrite reactions yielded two to three ultraviolet-absorbing compounds as major products depending on the reaction conditions used. Figure 3 represents the chromatographic patterns observed for reactions under various conditions. One of the major products (designated (I) in Fig. 3) was isolated in a crystalline form by preparative thin-layer chromatography, and identified as an oxime nitrite of sorbic acid (structure I; see below for the identification). Another product (designated x in Fig. 3) was a yellow oil, the structure of which is unknown.

It was also found that compound I could be prepared on a large scale by either (a) treatment of sorbic acid in chloroform with N_2O_3, or (b) treatment of sorbic acid with nitrite in HCl–acetic acid mixture followed by extraction of the products with ether and precipitation of the oxime nitrite with chloroform.

Identification and properties of the oxime nitrite of sorbic acid (I)

The material obtained by extensively recrystallizing crude compound I showed an elemental composition corresponding to an adduct formed between sorbic acid and N_2O_3. The mass spectrum of this compound showed a molecular peak of the adduct. In view of the previously reported nitroso-nitrite formations from olefins and nitrous acid (Park & Williams, 1969), the possible structures for this product were the nitroso-nitrites, or their tautomeric forms, i.e. oxime nitrites. In addition, these compounds can result either from 1,2- or 1,4-addition of N_2O_3 across the conjugated double bonds of sorbic acid. The n.m.r. signals of this compound in the 1–7 ppm region indicated that there were six protons attached to carbons. This eliminated the possibility of the nitroso-nitrite structures in which seven protons must be present on the carbons. From the spectrum, the structure was assigned as I (Fig. 1).

The oxime nitrite before the extensive crystallization was a mixture of two isomers, the *anti* and *syn* conformations of the oxime group. Evidence for the isomeric nature of the two compounds was as follows: (1) the mixture gave an elemental composition identical with that of the purified oxime nitrite; (2) the n.m.r. signals of the minor component were almost identical with those of the major component (the pure compound) regarding their chemical shifts; (3) only single spots were detected on the thin-layer chromatograms of the mixture, using four different solvent systems; (4) the infrared spectrum of the mixture was virtually identical with that of the purified material; (5) when the purified species of the oxime nitrite was treated with alkali and the oxime nitrite was re-isolated, the material obtained was again a mixture of the two isomers.

Structures of the two conformers for I are shown in Fig. 1. The fact that the n.m.r. signal for the proton at position 2 of the minor component is located at considerably lower field than that of the major component, may be interpreted as resulting from an interaction of the proton with the *syn* oxime group. This interpretation leads to the assignment that the major and minor components are Ia (*anti*) and Ib (*syn*), respectively.

When the oxime nitrite I was allowed to stand in 0·1 M-HCl at 37°C, it was gradually converted to a new compound. This process showed first-order kinetics with an observed k value of $2{\cdot}7 \times 10^{-3}$ min^{-1}

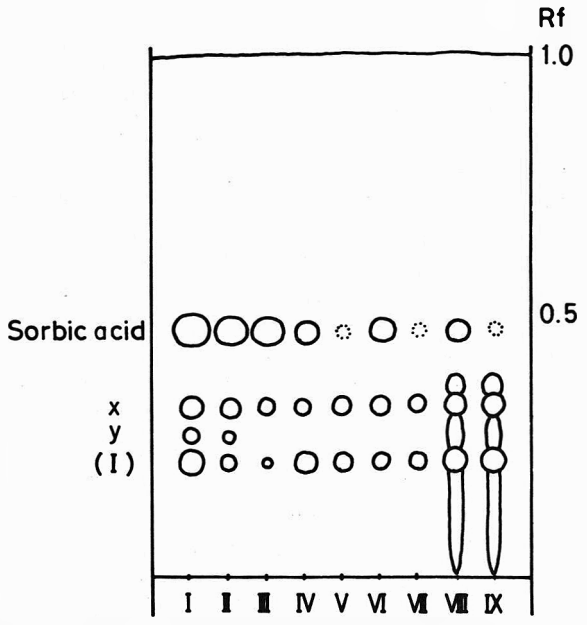

Fig. 3. Thin-layer chromatograms of the products of reacting nitrite and sorbic acid under the conditions summarized in Table 1 using *n*-hexane-dioxane-acetic acid as solvent.

($t_{0.5}$ = 260 min). The rate of the conversion was not affected by the presence of nitrite in the mixture. The product was isolated as crystalline material, which possessed an elemental composition corresponding to a dehydrated structure of the oxime nitrite I. Based on n.m.r. and ultraviolet spectra, the compound was tentatively assigned the closed-ring structure II (Fig. 1). The unidentified major product of the sorbic acid–nitrite reaction (compound x in Fig. 3) was different from this compound in its behaviour in silica-gel thin-layer chromatography.

Inhibition of in vitro *nitrosamine formation by sorbic acid*

Since sorbic acid reacted easily with nitrite, it was expected that sorbic acid would inhibit the *in vitro* formation of nitrosamines from amines and nitrite. The amines used were dimethylamine, morpholine and *N*-methylaniline, which react with nitrite with low (Mirvish, 1970), moderate (Fan & Tannenbaum, 1973), and high (Kalatzis & Ridd, 1966) reactivities respectively.

The inhibition of the *N*-nitrosodimethylamine formation by sorbic acid was studied using several combinations of reactant concentrations and pH values. For comparison, the inhibitory action of ascorbic acid was also tested under the same conditions. As Table 2 shows, the ability of sorbic acid to inhibit the *N*-nitrosodimethylamine formation was approximately equal to that of ascorbic acid in every experiment performed. In a separate experiment, *N*-nitrosodimethylamine was treated with sorbic acid at pH 3 and 37°C, and no degradation of the nitrosamine was observed.

N-Nitrosomorpholine formation was also inhibited by sorbic acid (Table 3). However, the inhibition was weaker than that effected by ascorbic acid. The very fast nitrosation of *N*-methylaniline was not inhibited by sorbic acid, while it was strongly inhibited by ascorbic acid (Table 3).

Tests for mutagenic activity in sorbic acid–nitrite reaction products

Compound I and the oily unidentified product (x in Fig. 3) were examined for mutagenic activity in *S. typhimurium* test strains TA98, TA100 and TA1538. Neither of these compounds showed positive results in these test systems either with or without metabolic activation. Negative results were also obtained in tests of these compounds for their DNA-damaging activity using a recombination-deficient *B. subtilis* test strain. The reaction mixture itself (10 mM-sorbic acid and 100 mM-$NaNO_2$, pH 1, 37°C) was tested for mutagenicity, and did not give positive results within the 2-hr reaction period. No metabolic activation was attempted in this case.

DISCUSSION

Angeli (1893) described a reaction of sorbic acid with N_2O_3 in ether resulting in an adduct of composition $C_6H_8N_2O_5$. Our present study has confirmed Angeli's observation, established the structure and shown that the same product can be formed by reaction of sorbic acid with nitrite in acidic media.

The concentrations of the reactants used in the studies of the reaction between sorbic acid and nitrite are considerably below the upper limits legally permitted in food in Japan (2000 ppm sorbic acid in meats, and 70 ppm nitrite in some meats). It is possible, therefore, that the reaction observed in the *in vitro* system does occur *in vivo* when these reagents are simultaneously ingested by man.

The reactivity of sorbic acid with nitrite is consistent with the finding that sausages containing sorbic acid have a decreased nitrite content (Nagata & Ando, 1971).

We have previously reported that a reaction between high concentrations of sorbic acid and nitrite results in the formation of mutagenic compounds (Hayatsu, Chung, Kada & Nakajima, 1975). The conditions under which the mutagenic products were formed were those given for lane IX (Table 1). It can be seen that in such stringent conditions, subsidiary products are formed in addition to the major products. In fact, one of the side products has been isolated as a crystalline compound and shown to be 2,5-dinitro-5-chloropenta-2,4-diene (K. C. Chung, H. Hayatsu, T. Kada, Y. Urata and Y. Iitaka, unpublished work 1977). It has also been shown that ethylnitrolic acid, a compound that can interact with DNA, was formed when a solution of sorbic acid and nitrite was heated (Namiki & Kada, 1975).

The fact that sorbic acid reacts with nitrite presents potential danger at high concentrations although the inhibition of nitrosamine formation, as demonstrated *in vitro* in the present paper, appears to be more realistic.

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ENHANCED PESTICIDE METABOLISM, A PREVIOUSLY UNREPORTED EFFECT OF DIETARY FIBRE IN MAMMALS*

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Abstract—The effects of various dietary fibres on the metabolism of the organochlorine insecticide, lindane, were compared. Groups of six weanling female Sprague–Dawley rats were fed either a synthetic low-residue diet (LRD), LRD + 10% pectin, LRD + 10% agar, LRD + 10% cellulose, or Purina Lab Chow for 28 days. The animals were then dosed orally with 2·87 mg lindane (containing 1·66 μCi [U-^{14}C]lindane) and were killed 24 hr later. A smaller proportion of administered radioactivity was recovered in the excreta and selected tissues from the rats fed the LRD diet than from other groups and the fate of the radioactivity not accounted for was investigated in a second experiment using rats fed either LRD unsupplemented, LRD + 10% pectin or the standard chow diet. Pectin and the dietary fibre contained in Purina Lab Chow caused significant alterations in the metabolism of lindane. A significant increase in the excretion of radiolabelled products, a higher level of conjugated chlorophenols and polar metabolites, a significant alteration in the proportions of the excreted chlorophenols and significant stimulation of the enzymes involved in lindane metabolism indicated that dietary fibre such as pectin or the plant fibre in Purina Lab Chow can significantly affect the metabolism of xenobiotics in mammals.

INTRODUCTION

Dietary fibre has been shown to counteract the toxicity resulting from exposure to oestrogens (Ershoff, 1964), glucoascorbic acid (Ershoff, 1954 & 1957), Tween 60 (Ershoff & Hernandez, 1959; Ershoff & Marshall, 1975), chlorazanil hydrochloride (Ershoff, 1959), the red dye, amaranth (Ershoff & Thurston, 1974), sodium cyclamate (Ershoff, 1972), 2-acetylaminofluorene (Engel & Copeland, 1952), azoxymethane (Ward, Yamamoto & Weisburger, 1973), polychlorinated biphenyls (Kiriyama, Inoue, Machinaka & Yoshida, 1973), DDT (Kiriyama *et al.*, 1973), heavy metals (Arkhipova & Zorina, 1965) and X-rays (Ershoff, Bajwa, Field & Bavetta, 1969; Ershoff, Bajwa, Shapiro & Bernick, 1967). Little information is available regarding the mechanism by which plant fibres exert their protective effect, but it appears to be unrelated to bulk-forming capacity or cellulose content *per se* (Ershoff, 1974). Moreover, marked differences in the protective properties of various dietary fibres have been reported (Ershoff, 1974; Ershoff & Marshall, 1975). Up to this time, the effect of dietary fibre on the metabolism of xenobiotics has received little attention. This study reports the comparative effects of various dietary fibres on the storage, excretion and metabolism of the organochlorine insecticide γ-hexachlorocyclohexane (lindane; γ-BHC).

EXPERIMENTAL

Thirty weanling female Sprague–Dawley rats were housed in individual metabolism cages and assigned to one of five dietary groups. One group received Purina Laboratory Chow (Ralston-Purina Co., Inc., St. Louis, MO) throughout the study. The remaining four groups were fed Normal Protein Test Diet, a synthetic low-residue diet obtained from Nutritional Biochemicals, Cleveland, OH. For 28 days prior to treatment with lindane (obtained from the Health Effects Research Laboratory Analytical Reference Standards Repository, Research Triangle Park, NC), the rats were fed Purina Laboratory Chow or the low-residue diet either unsupplemented or supplemented with 10% agar, with 10% Alphacel or with 10% citrus pectin (all three obtained from Nutritional Biochemicals). All animals were then dosed orally with 2·87 mg lindane containing 1·66 μCi [U-^{14}C]lindane (specific activity 48 mCi/mmol, radiochemical purity 99%; from Amersham/Searle Corp., Arlington Heights, IL) and were killed 24 hr later.

Urine, faeces, fat, liver, kidney and serum samples were taken for analysis of radioactivity. Liver, kidney, fat, serum and faeces samples were subjected to combustion in a Packard Tri-Carb Model 306 sample oxidizer and the radioactivity was determined in a Packard Tri-Carb Model 3380 liquid scintillation spectrometer. The hepatic content of microsomal protein (Lowry, Rosebrough, Farr & Randall, 1951) and of cytochrome *P*-450 (Omura & Sato, 1964a,b) and the enzyme activity involved in the dehydrogenation and dechlorination of lindane (Chadwick, Chuang & Williams, 1975) were determined. Individual 24-hr urine samples were collected and extracted and the extracts were analysed on a Tracor Model MT-220 gas chromatograph equipped with an electron-capture detector and a Coulson electrolytic conductivity detector as previously described (Chadwick, Simmons, Bryden, Chuang, Key & Chadwick, 1977). Faecal samples were also analysed for moisture content.

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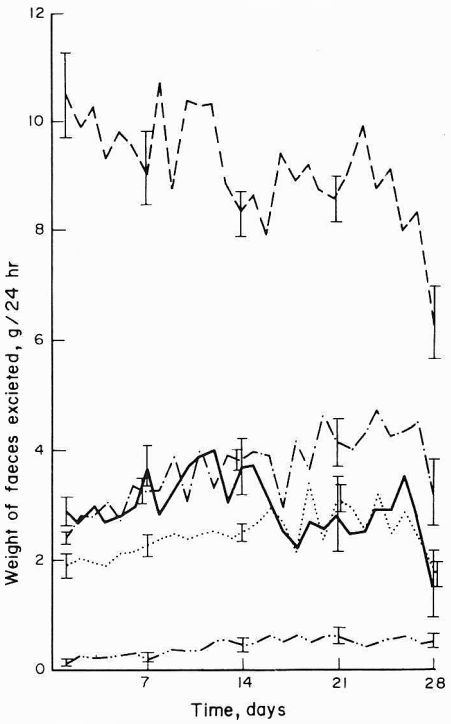

Fig. 1. Effect of dietary fibre on the daily excretion of faeces by rats fed a low-residue diet (LRD; —·—), LRD + 10% pectin (—·—), LRD + 10% agar (·····), LRD + 10% Alphacel (——) or regular lab chow (– – –). Values are means for groups of six rats, the SEM being indicated by vertical lines at 1, 7, 14, 21 and 28 days.

To account for radioactivity not recovered from the tissues taken in the first study, another experiment was initiated in which 18 weanling female Sprague–Dawley rats were randomly assigned to one of three dietary groups and fed either the unsupplemented low-residue diet, the low-residue diet containing 10% pectin or Purina Laboratory Chow. Experimental conditions, duration of treatment and analytical procedures were the same as those used in the first experiment, except that after treatment with lindane, the rats were transferred to animal containment chambers (Plas-Labs, Lansing, MI) and the expired air was passed through Carbo-sorb II (Packard, Downers Grove, IL) for $^{14}CO_2$ collection (Stevens, Hall, Farmer, DiPasquale, Chernoff & Durham, 1977). Urine, faeces, expired air, brain, thymus, lung, heart, liver, kidneys, adrenals, spleen, stomach, small intestine, large intestine, ovaries, bladder, fat, muscle and serum were analysed for radioactivity. Organs were weighed at autopsy.

Body weight, food consumption and urinary and faecal excretion were measured daily throughout the experiments. The energy content of the diet and of faecal samples was determined by combustion in a calorimetric bomb containing oxygen under pressure (ASTM, 1975).

Student's *t* test (Snedecor, 1956) and Duncan's multiple range test (Duncan, 1955) were used as aids in the interpretation of the data from this study. Differences were considered significant at $P < 0.05$ unless otherwise stated.

RESULTS

In the first experiment, the mean daily faecal excretion of all animals receiving fibre was significantly higher throughout the study than that of the rats fed the unsupplemented low-residue diet (Fig. 1). Moreover, the mean daily faecal excretion of the rats fed Purina Laboratory Chow was significantly higher than that of the other rats in the study.

Food consumption and gross energy intake were significantly higher in the rats fed the Purina Lab Chow than in the other rats, yet because of the energy lost in the excreted faeces, these animals had significantly less energy available for weight gain than all the other rats except those fed the low-residue diet supplemented with agar (Table 1). On the other hand, the rats fed the unsupplemented low-residue diet, although showing the lowest values for food consumption and gross energy intake, had high food-conversion efficiencies and high digestion coefficients, and thus had significantly more energy available for weight gain than did rats fed the Purina Lab Chow.

Figure 2 shows that while neither body nor kidney weights differed significantly, the livers of the rats fed Purina Lab Chow were significantly larger than those of the other animals. Moreover, the rats fed Purina Lab Chow had significantly higher enzyme activity *in vitro* for both the dehydrogenation and dechlorination of lindane than all other rats in the study. Hepatic cytochrome *P*-450 content of the rats fed Purina Lab Chow was significantly higher than all other rats except those fed the low-residue diet supplemented with 10% pectin. All animals fed diets containing fibre had significantly higher dechlorinase activity than the rats fed the unsupplemented low-residue diet. There were no significant differences in microsomal-protein content.

Total radioactivity in the urine and faeces of rats fed the Purina Lab Chow was significantly higher than that excreted by the other groups, while that from the rats fed the low-residue diet supplemented with pectin was significantly greater than the radioactivity excreted by animals on the unsupplemented low-residue diet or the low-residue diet containing Alphacel (Table 2). However, urine from the rats fed Purina Lab Chow had a significantly higher concentration of radioactivity than that of the other animals, whereas faeces from the rats receiving the low-residue diet supplemented with pectin contained the highest concentration of faecal radioactivity (Table 2). Moreover the distribution of radioactivity indicated that while there were no significant differences in the urinary excretion of neutral metabolites, the rats fed Purina Lab Chow excreted significantly more chlorophenols and polar metabolites than the other animals (Table 3). In addition, the rats fed the low-residue diet containing pectin excreted significantly more conjugated chlorophenols and polar metabolites in the urine than did the animals on the unsupplemented low-residue diet (Table 3).

Although there was no significant difference in the renal excretion of the neutral metabolites, trace amounts of lindane, 2,3,4,5,6-pentachloro-2-cyclohexen-1-ol and 2,3,4,6-tetrachloro-2-cyclohexen-1-ol were detected by gas chromatography.

Feeding either regular lab chow or the low-residue

Table 1. *Effect of dietary fibre on food consumption and energy intake of female rats*

Parameter	Values (means + SEM) for groups of six rats fed diet no.*					Comparisons†
	(1)	(2)	(3)	(4)	(5)	
Food consumption (g/28 days)	345 ± 12·6	400 ± 20·3	387 ± 15·4	410 ± 11·6	514 ± 10·4	5 > all; 4, 2, 3 > 1
Food conversion efficiency‡	0·30 ± 0·01	0·28 ± 0·01	0·29 ± 0·01	0·28 ± 0·01	0·23 ± 0·01	All > 5
Energy intake (kcal/rat/28 days):						
gross	1548 ± 56·73	1755 ± 88·92	1730 ± 68·78	1774 ± 50·12	2050 ± 41·70	5 > all; 4, 2 > 1
digestible§	1512 ± 54·68	1567 ± 84·02	1494 ± 56·86	1550 ± 45·36	1439 ± 43·18	NS
Digestion coefficient‖	97·7 ± 0·18	89·2 ± 1·30	86·3 ± 0·32	87·3 ± 0·28	70·2 ± 1·05	1 > all; 2, 4, 3 > 5; 2 > 3
Energy available for gain (kcal/rat/day)**	25·8 ± 1·36	27·2 ± 2·17	24·2 ± 1·14	25·4 ± 1·10	20·3 ± 1·20	2, 1, 4 > 5

NS = Not significant

*(1) Low-residue diet (LRD); (2) LRD ± 10% pectin; (3) LRD ± 10% agar; (4) LRD ± 10% Alphacel; (5) Purina Lab Chow.

†Comparisons reported differ significantly ($P < 0·05$) by Duncan's multiple range test.

‡Food conversion efficiency = weight gained (g)/diet consumed (g).

§Digestible energy intake = gross energy intake − energy excreted in faeces.

‖Digestion coefficient = digestible energy intake × 100/gross energy intake.

**Energy available for gain = digestible energy intake in 24 hr − (body weight $(kg)^{3/4}$ × 115 kcal).

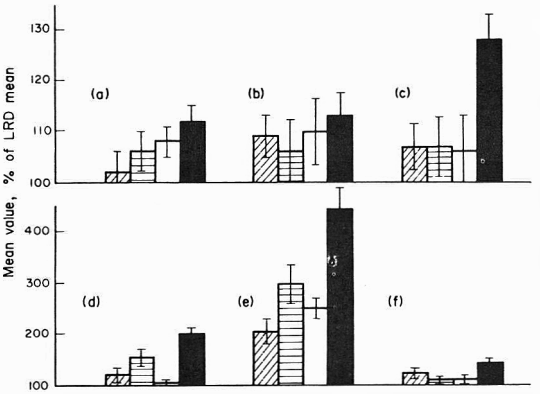

Fig. 2. Effect of dietary fibre on (a) body weight, (b) kidney weight, (c) liver weight, (d) the dehydrogenation of lindane, (e) the dechlorination of lindane and (f) the hepatic cytochrome *P*-450 content. The mean value for the six rats on the low-residue diet (LRD) is represented as 100%, the mean values of the other dietary groups being presented as a percentage of the LRD value (dietary mean × 100/ LRD mean). Vertical lines indicate the SEM and in each block the bars indicate the values for the groups of six rats fed LRD + 10% pectin (▨), LRD + 10% agar (▤), LRD + 10% Alphacel (□) and regular lab chow (■).

diet plus 10% pectin significantly altered the relative proportions of the urinary tetrachlorophenol metabolites (Fig. 3). While rats on either of these diets excreted a significantly higher proportion of 2,3,4,6-tetrachlorophenol than the other animals, the rats fed Purina Lab Chow excreted significantly more of this compound that those fed the low-residue diet containing pectin. On the other hand, all four groups on the low-residue diet excreted a significantly higher proportion of 2,3,4,5-tetrachlorophenol than did those fed Purina Lab Chow. Moreover the rats fed the low-residue diet plus pectin excreted a significantly smaller proportion of 2,3,4,5-tetrachlorophenol than did those fed the low-residue diet or the low-residue diet plus Alphacel.

Since rats fed the low-residue diet excreted significantly less radioactivity than those fed Purina Lab Chow or the low-residue diet supplemented with pectin, it was assumed that the animals consuming the low-residue diet were storing the radiolabelled lindane. However, no significant differences between the groups were found in serum, liver, kidney or fat levels of radioactivity. To account for the radioactivity not recovered from the excreta or tissues of the rats on the unsupplemented low-residue diet, a second experiment was initiated using the unsupplemented low-residue diet, the low-residue diet supplemented with 10% pectin and Purina Lab Chow. This time urine, faeces, expired air, brain, thymus, lung, heart, liver, kidneys, adrenals, spleen, stomach, small intestine, large intestine, ovaries, bladder, fat, muscle and serum were analysed for radioactivity.

Again, feeding either regular lab chow or the low-residue diet containing pectin resulted in a significant increase in the total excretion of radioactivity over that of the animals on the unsupplemented low-residue diet (Table 4). In this second experiment, a marked decrease in the 24-hr faecal collection was noted for all dietary groups. This may have been due to stress resulting from the transfer of the animals to a different type of cage 24 hr before they were killed. Thus, although the radioactivity in the faeces of the rats fed Purina Lab Chow was again significantly greater than that of the rats fed the low-residue diet, the radioactivity in the faeces of the rats fed the low-residue diet containing pectin was not. Small amounts of radioactivity were expired by all dietary groups (Table 4), the amount expired both by the rats fed Purina Lab Chow and by those fed the low-residue diet supplemented with pectin being significantly greater than that recovered from the animals fed the unsupplemented low-residue diet.

Only the tissues listed in Table 4 contained significantly differing levels of radioactivity in the three groups. In all cases, the rats on the low-residue diet had the lowest level of radiolabelled material. It was noted, however, that the organ weights of the animals on the low-residue diet were lower and therefore a comparison of total organ weight as a percentage of body weight was made (Table 5). Organs of the rats fed the unsupplemented low-residue diet comprised 13·6% of the total body weight, a proportion significantly lower than the 17 and 19% found in the rats fed the low-residue diet containing pectin or Purina Lab Chow, respectively.

DISCUSSION

Dietary fibre is not just an inert substance that passes unchanged through the gut. It consists of a heterogeneous group of compounds, including cellulose, polysaccharides, pectic substances, hemicelluloses and lignins, with distinct physical, chemical and metabolic properties (Cummings, 1973; Trowell, 1972). The amounts of these components vary according to the type of plant and its anatomy. While the water-adsorption capacity of cellulose is moderate, that of the other polysaccharides is strong. Lignin, a hydroxylated aromatic polymer, absorbs acid compounds but not significant amounts of water. Pectic substances form gels with water and cause dilution of faecal constituents (Williams & Olmsted, 1936).

In accordance with these findings, the diets used in this study, a low-residue diet, unsupplemented or supplemented with 10% pectin, 10% agar or 10% Alphacel, and Purina Lab Chow, resulted in faeces with moisture contents of 23·5, 48·0, 38·1, 19·0 and 39·2% respectively. Moreover, natural plant fibre was shown to bind significantly more bile salts than non-nutritive fibre such as cellophane, spangles and cellulose (Kritchevsky & Story, 1974). Further evidence that dietary fibre is not inert comes from reports that dietary-fibre constituents from molasses (Fahey, Williams & McLaren, 1976) and from corn, alfalfa and spinach (McLaren, Cuppett, Williams, Fahey & Smith, 1974) are growth stimulants. Evidence for the involvement of dietary fibre in metabolism includes reports that it stimulates the activity of hepatic cholesterol 7α-hydroxylase, a cytochrome *P*-450 enzyme (Johansson, 1970; Morgan, Heald, Atkin & Green, 1974). However, fibre-induced acceleration of toxicant metabolism, such as that observed in this study, has not been previously reported and may constitute a mechanism whereby dietary fibre exerts a protective effect against the toxicity of some xenobiotics.

A significantly greater excretion of radiolabelled lindane metabolites (Tables 2 & 4), a significant increase in the metabolism of lindane to polar metabolites

Table 2. *Effect of diet on excretion of radioactivity after treatment of female rats with* [^{14}C]*lindane*

Radioactivity excreted	Values (means + SEM) for groups of six rats fed diet no.*					Comparisons†
	(1)	(2)	(3)	(4)	(5)	
Total (dpm × 10^{-5})	3·09 ± 0·20	4·85 ± 0·83	3·57 ± 0·12	3·38 ± 0·24	6·25 ± 0·25	5 > all
(% of dose)	8·44 ± 0·54	13·20 ± 2·25	9·76 ± 0·32	9·24 ± 0·65	17·10 ± 0·69	2 > 4, 1
In urine (dpm × 10^{-5})	2·87 ± 0·14	3·55 ± 0·36	3·19 ± 0·14	3·09 ± 0·24	4·74 ± 0·16	5 > all
(% of dose)	7·85 ± 0·39	9·70 ± 0·99	8·72 ± 0·39	8·45 ± 0·66	13·00 ± 0·42	
(dpm × 10^{-5}/ml)	0·057 ± 0·003	0·071 ± 0·007	0·064 ± 0·003	0·062 ± 0·005	0·095 ± 0·003	5 > all 2 > 1
In faeces (dpm × 10^{-5})	0·26 ± 0·10	1·30 ± 0·51	0·38 ± 0·17	0·29 ± 0·08	1·51 ± 0·18	5, 2 > 3, 4, 1
(% of dose)	0·72 ± 0·26	3·55 ± 1·40	1·04 ± 0·19	0·80 ± 0·23	4·12 ± 0·50	
(dpm × 10^{-5}/g)	0·71 ± 0·16	1·87 ± 0·82	0·20 ± 0·04	0·18 ± 0·04	0·40 ± 0·03	2 > 5, 4, 3

*(1) Low-residue diet (LRD); (2) LRD ± 10% pectin; (3) LRD + 10% agar; (4) LRD + 10% Alphacel; (5) Purina Lab Chow.
†Comparisons reported differ significantly ($P < 0·05$) by Duncan's multiple range test.

Table 3. *Effect of diet on the distribution of radioactivity in the urine of female rats treated with* [^{14}C]lindane

Identity of metabolites	Urinary radioactivity (dpm × 10^{-5}) as means ± SEM for groups of six rats on diet no.*					Comparisons†
	(1)	(2)	(3)	(4)	(5)	
Neutral metabolites‡	0·094 ± 0·035	0·078 ± 0·006	0·086 ± 0·020	0·052 ± 0·004	0·098 ± 0·012	NS
Free chlorophenols§	0·74 ± 0·04	0·80 ± 0·04	0·80 ± 0·05	0·81 ± 0·05	1·32 ± 0·10	5 > all
Conjugated chlorophenols‖	0·92 ± 0·04	1·25 ± 0·12	1·10 ± 0·07	1·07 ± 0·06	1·76 ± 0·10	5 > all 2 > 1
Polar metabolites**	0·97 ± 0·04	1·26 ± 0·11	1·09 ± 0·03	1·08 ± 0·05	1·64 ± 0·07	5 > all 2 > 1

NS = Not significant
*(1) Low-residue diet (LRD); (2) LRD ± 10% pectin; (3) LRD ± 10% agar; (4) LRD ± 10% Alphacel; (5) Purina Lab Chow.
†Comparisons reported differ significantly ($P < 0·05$) by Duncan's multiple range test.
‡Extracted with benzene from acid urine (pH = 2) but did not partition into 0·15 N-NaOH.
§Extracted with benzene from acid urine (pH = 2) and partitioned into 0·15 N-NaOH.
‖Extracted with benzene from hydrolysed urine.
**Remained in residual hydrolysed urine after extraction.

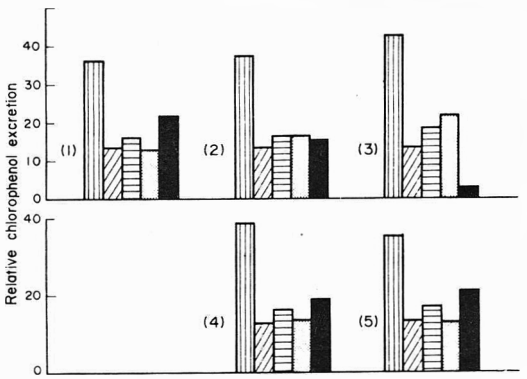

Fig. 3. Effect of dietary fibre on the excretion patterns of the chlorophenol metabolites of lindane in groups of six rats fed (1) low-residue diet (LRD), (2) LRD + 10% pectin, (3) regular lab chow, (4) LRD + 10% agar or (5) LRD + 10% Alphacel. Each bar is the mean for six animals and shows, for each dietary group, the percentage of the total chlorophenol excretion represented by (▥) 2,4,6-, (▨) 2,3,5- and (▤) 2,4,5-trichlorophenol and (▩) 2,3,4,6- and (■) 2,3,4,5-tetrachlorophenol.

and conjugated chlorophenols (Table 3), a significant alteration in the relative proportions of the excreted chlorophenol metabolites of lindane (Fig. 3) and significant stimulation of the enzymes involved in lindane metabolism (Fig. 2) support the contention that a dietary fibre such as pectin or the variety of plant fibres in Purina Lab Chow can affect the metabolism of lindane. Total dry weight of the faeces excreted by rats fed Purina Lab Chow equalled 31·5% of the quantity of food consumed by these animals during the experiment and this proportion was significantly higher than that of the other rats in the study. This indicates that the dietary fibre content of purina Lab Chow is significantly greater than that of the other diets. Although the significantly high level of dietary fibre in Purina Lab Chow is apparently related to the significantly increased lindane metabolism in animals fed this diet, other factors, such as the type of dietary protein, lipid or trace contaminants could have contributed to the increased metabolism. Such factors, however, are not involved in the increased lindane metabolism noted in rats fed the low-residue diet supplemented with pectin.

Table 4. *Effect of diet on radioactivity excreted and retained in the tissues after treatment of female rats with [^{14}C]lindane*

Radioactivity excreted or retained	Values for rats fed diet no.†		
	(1)	(2)	(3)
Total (dpm × 10^{-5})	2·78 ± 0·28	3·88 ± 0·38**	5·10 ± 0·45**
(% of dose)	7·40 ± 0·85	10·20 ± 1·01	13·60 ± 1·35
In urine (dpm × 10^{-5})	2·72 ± 0·28	3·73 ± 0·39*	4·36 ± 0·45**
(% of dose)	7·24 ± 0·84	9·85 ± 1·05	11·60 ± 1·31
faeces‡ (dpm × 10^{-5})	0·006 ± 0·004	0·097 ± 0·061	0·580 ± 0·150**
(% of dose)	0·013 ± 0·010	0·247 ± 0·148	1·560 ± 0·410
breath (dpm × 10^{-5})	0·057 ± 0·006	0·069 ± 0·002*	0·156 ± 0·038**
(% of dose)	0·150 ± 0·017	0·181 ± 0·006	0·423 ± 0·106
brain§	0·40 ± 0·07	0·56 ± 0·06	0·70 ± 0·05**
liver§	3·86 ± 1·34	6·82 ± 0·98*	7·96 ± 0·89**
kidneys§	0·83 ± 0·18	1·20 ± 0·21	1·58 ± 0·15**
stomach§	1·03 ± 0·25	2·36 ± 0·77	4·57 ± 1·16**
large intestine§	7·62 ± 1·05	12·90 ± 2·65*	8·88 ± 0·98
muscle‖	0·22 ± 0·04	0·39 ± 0·06**	0·50 ± 0·07**

†(1) Low-residue diet (LRD); (2) LRD + 10% pectin; (3) Purina Lab Chow.
‡Two rats on diet (1) and one on diet (2) did not excrete faeces within the 24-hr period.
§Expressed as dpm × 10^{-4}/whole organ, or pair of kidneys.
‖Expressed as dpm × 10^{-4}/g tissue.
Values are means ± SEM for groups of six rats and those marked with asterisks differ significantly from the LRD values: *$P < 0·10$; **$P < 0·05$.

Table 5. *Effect of dietary fibre on body weight and the combined weight of the excised organs of female rats*

Parameter	Values for rats fed diet no.†		
	(1)	(2)	(3)
Body weight (g)	202 ± 8·91	201 ± 2·55	219 ± 7·71
Total organ weight‡ (g)	27·5 ± 1·59	34·1 ± 0·71*	42·0 ± 2·23*
Organ weight/body weight ratio	0·136 ± 0·003	0·170 ± 0·004*	0·192 ± 0·007*

†(1) Low-residue diet (LRD); (2) LRD ± 10% pectin; (3) Purina Lab Chow.
‡Organs excised and weighed comprised liver, kidneys, stomach, small intestine, large intestine, brain, thymus gland, heart, lungs, spleen, adrenal glands, bladder and ovaries.
Values are means ± SEM for groups of six animals and those marked with an asterisk differ significantly (Student's *t* test) from the LRD values: *$P < 0·05$.

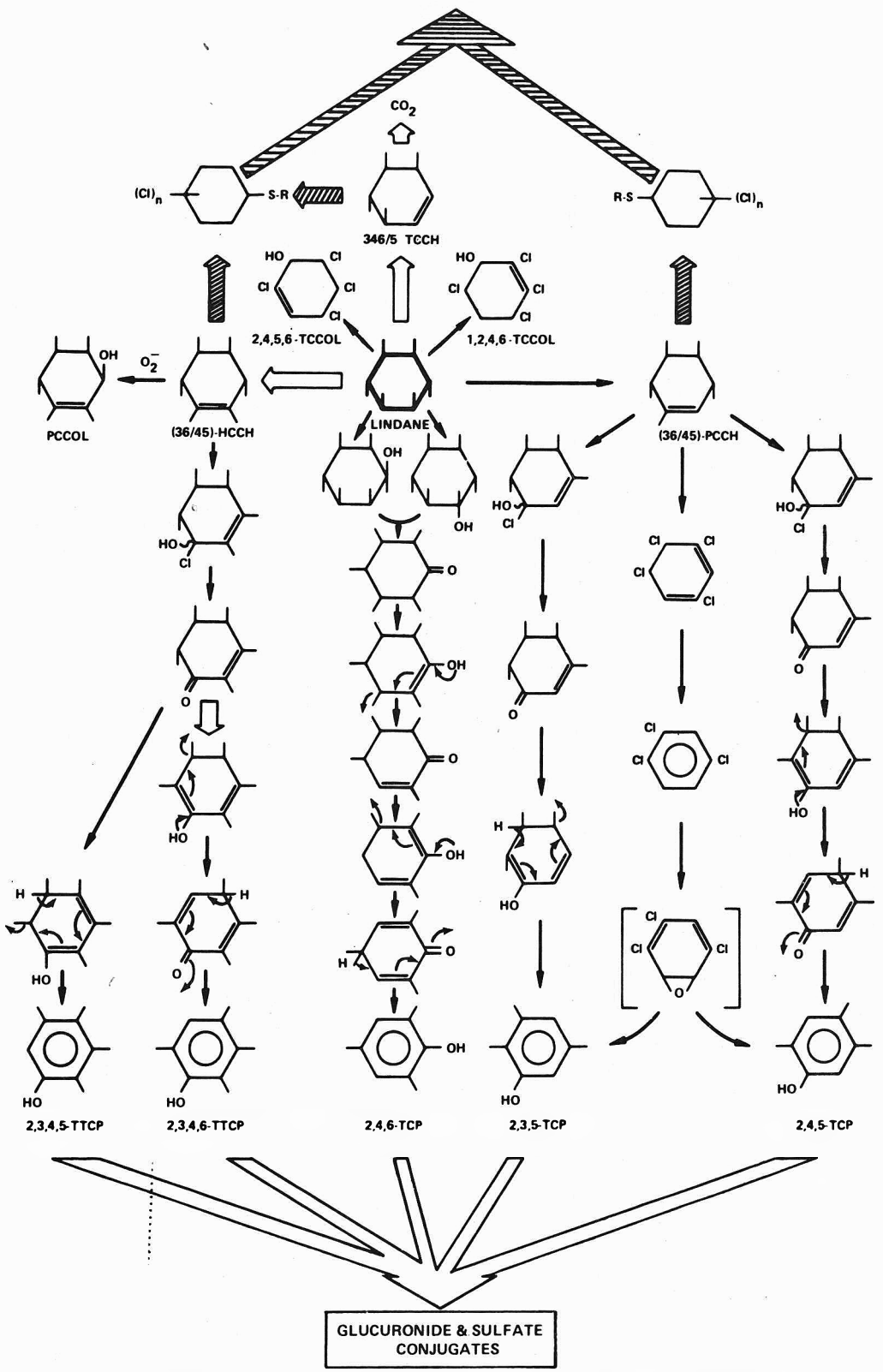

Fig. 4. Effect of dietary fibre on the metabolism of lindane. The open arrows indicate the metabolic pathways affected by dietary fibre in this study. The shaded arrows indicate pathways that may be influenced in view of the elevated excretion of radiolabelled polar lindane metabolites by rats fed Purina Lab Chow or low-residue diet + 10% pectin. Where the configurations of the compounds are known, this is indicated by fractional notation, numbers in the numerator denoting chlorine above and numbers in the denominator denoting chlorine below the plane of the ring. Abbreviations: TCCH, tetrachlorocyclohexene; TCCOL, tetrachlorocyclohexenol; PCCOL, pentachlorocyclohexenol; HCCH, hexachlorocyclohexene; PCCH, pentachlorocyclohexene; TTCP, tetrachlorophenol; TCP, trichlorophenol.

The observed effects of dietary fibre on the metabolism of lindane are summarized in Fig. 4, in which the open arrows indicate the metabolic pathways affected. The proposed mechanisms of lindane degradation in mammals are a composite of previous observations and current reports (Chadwick, Chadwick, Freal & Bryden, 1977; Chadwick *et al.*, 1975; Chadwick & Freal, 1972; Freal & Chadwick, 1973; Grover & Sims, 1965; Tanaka, Kurihara & Nakajima, 1976 & 1977).

The increased excretion of conjugated chlorophenols (Table 3) by rats fed either Purina Lab Chow or the low-residue diet supplemented with pectin may reflect the influence of dietary fibre on intestinal bacteria. Both rats and man carry anaerobic intestinal bacteria which are able to hydrolyse glucuronide and sulphate conjugates (Reddy, Weisburger & Wynder, 1974; Renwick & Draser, 1976) and the ratio of total anaerobes to aerobes is higher in western man than in societies with a high fibre intake (Aries, Crowther, Drasar, Hill & Williams, 1969; Hill, Crowther, Drasar, Hawksworth, Aries & Williams, 1971).

The significantly higher dechlorination of lindane to 3,4,5,6-tetrachlorocyclohexene (Fig. 2) accompanied by a significantly increased expiration of labelled material (Table 4) in rats fed pectin or the plant fibres in Purina Lab Chow indicates that dietary fibre may affect a metabolic pathway similar to one reported in micro-organisms (Matsumura, Berezet & Patil, 1976). The latter group demonstrated that micro-organisms such as *Pseudomonas putida* dechlorinate lindane to 3,4,5,6-tetrachlorocyclohexene and then further degrade this metabolite, by an FAD-dependent reductive dechlorination mechanism followed by ring opening and oxidation, to yield CO_2.

Since there were no significant differences in terminal body weight (Fig. 2; Table 6) and since the total organ weight of the rats fed the unsupplemented low-residue diet was significantly smaller (Table 6), it is postulated that the energy available for weight gain in these animals (Table 1) goes to body fat, a high-energy pool in mammals. It has been reported that the fat depots of rats on a purified corn-starch diet weighed twice as much as those of rats fed a whole-grain corn diet (Winnie, Schemmel, Rand, Mickelsen & Leveille, 1973). Thus although there were no significant differences in the concentration of radioactivity stored in the body fat of animals in this study, extensive storage in larger fat depots could account for the radioactivity that was neither excreted (Tables 2, 4) nor stored (Table 5) in the organs of the rats fed the unsupplemented low-residue diet.

In summary, dietary fibre can significantly alter the metabolism of lindane and presumably other environmental toxicants. Because of different physical, chemical and metabolic properties, however, some dietary fibres, such as pectin and the plant fibres in Purina Lab Chow, are more effective than others, such as agar and cellulose.

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NEPHROTOXIC AND HEPATOTOXIC EFFECTS OF DICHLOROACETYLENE

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Abstract—Rabbits were exposed to inhalation of dichloroacetylene (DCA) in lethal and sublethal doses for 1 hr (126, 202 and 307 ppm) or 6 hr (17–23 ppm) to identify pathomorphological kidney and liver lesions and to demonstrate characteristic enzyme and electrolyte changes in the serum. DCA was potently nephrotoxic, the severe azotaemia demonstrated correlating with extensive tubular necrosis and focal necrosis in the collecting tubes of the kidney. An increase in mitotic activity in the renal epithelium, disturbance of mitosis and an occasional striking enlargement of nuclei were present after 3 days, after which tubular necrosis was often associated with distinct calcification and a reactive connective-tissue proliferation with formation of collagenous fibres. Death following exposure to lethal concentrations of DCA was usually due to acute renal failure. The principal feature of liver damage at the time of death was fatty degeneration of the parenchyma, mainly involving the acinar periphery and characterized by medium to large lipid droplets. The serum transaminases showed only an initial transient elevation. After sublethal concentrations, uraemia reached its maximum 3 days after the exposure. Renal damage in man after DCA poisoning has not yet been reported.

INTRODUCTION

Dichloroacetylene (DCA) is a liquid known to be extremely reactive chemically. It decomposes rapidly in mixtures with air. Most DCA intoxications are due to inadequate handling of trichloroethylene and to occupational exposure to vinylidene chloride copolymers (Henschler, Broser & Hopf, 1970). According to a large number of published case histories, the main signs of poisoning in man are neurological disorders, predominantly of the trigeminal nerves. Animal experiments in rats, mice and rabbits, however, have identified the kidney as the main target organ (Reichert, Brinke, Bannasch & Liebaldt, 1972; Reichert, Ewald & Henschler, 1975). Long-term exposure of rats to lower concentrations of DCA have also demonstrated kidney involvement in the form of enlargement of cells and cellular nuclei in the renal tubules (Jackson, Lyon & Siegel, 1971; Siegel, Jones, Coon & Lyon, 1971). We report here further studies of the nephrotoxic and hepatotoxic activity of DCA, designed to clarify the type and time course of these lesions and their contribution to the pathological pattern of DCA intoxication.

EXPERIMENTAL

Animals and treatment. Male rabbits (white New Zealanders) weighing 2·5–3·5 kg (bred by P. Bäumler, D-8190 Wolfratshausen) were given water and standard diet (Altromin®, from Altromin GmbH, Lippe) *ad lib.* and were kept in an air-conditioned room with an artificial 12-hr day/night rhythm. The preparation and analytical control of the air–DCA mixture have been described elsewhere (Reichert *et al.* 1975), as have the exposure chamber for the rabbits and the results of 1-hr exposure experiments (Reichert, Liebaldt & Henschler, 1976).

Blood chemistry. Blood specimens were collected from the rabbits' ears and centrifuged at 2500 *g* for 10 min. An autoanalyser (SMA 1230, Technicon, Frankfurt/Main) was used to determine serum cholesterol, calcium, inorganic phosphate, bilirubin, albumin, total protein, uric acid, blood urea nitrogen (BUN), glucose, lactate dehydrogenase (LDH), alkaline phosphatase, and glutamic–oxalacetic transaminase (SGOT). Serum glutamic–pyruvic transaminase (SGPT) was determined in the Eppendorf Enzymautomat of the laboratories of the Medizinische Universitätspoliklinik, Würzburg.

Histology. Tissue sections were fixed in Carnoy's mixture or 10% formalin before they were embedded in paraffin. Paraffin sections were stained with haematoxylin and eosin, tri-PAS (counter-stained with Orange G and Weigert's haematoxylin), cresyl violet, Alcian Blue 8GS or colloidal iron. Fat red staining was performed with frozen sections. Acid mucopolysaccharides were demonstrated by colloidal iron staining, using the iron-binding reaction of Hale as modified by Graumann & Clauss (1958). Alcian blue staining of mucopolysaccharides was carried out according to Gomori (1954). To visualize precipitates of calcium carbonate in the kidney, the van Kossa reaction was used. PAS-positive material was characterized by the diastase reaction.

RESULTS

Morphological lesions in lethal DCA intoxication

Exposure to a high dose of DCA (307 ppm for 1 hr) was intended to disclose the cause of death and to give information on the time course of any injury and the target organ(s). Three of the nine exposed rabbits died during the first 2 days, and the surviving animals were killed in groups of 1–3 animals after 48, 96 and 168 hr.

After 48 hr, the kidney sections showed extensive tubular necrosis (Plate 1) and focal necrosis of the collecting tubules. Intact epithelium in the tubules and collecting tubules frequently showed PAS-positive granules, which were partly resistant to diastase. At this time, the liver sections were almost devoid of glycogen and a diffuse cytoplasmic basophilia (chromatolysis) had resulted from dispersal of basophilic bodies. Furthermore, pronounced fatty infiltration was located predominantly acinoperipherally. Parenchymal necrosis was very rarely encountered periportally.

After 96 hr the tubular necrosis in the kidney was still extensive. There was a significant increase in cytoplasmic basophilia in many preserved tubules, and there were numerous abnormal mitoses and, occasionally, strikingly large nuclei (Plate 2). Numerous PAS-positive glycogen granules, present in the collecting tubule epithelium, disappeared after pretreatment of the sections with diastase. The liver was characterized by a distinct acino-central storage of glycogen, combined in some areas with marked cytoplasmic acidophilia. There was only a minor periportal disappearance of glycogen, whereas the predominantly acino-peripheral fat deposition in the parenchymal cells was still pronounced.

After 168 hr, the renal tubular lesions still persisted over large areas. The damaged tubules were often calcified at this stage (Plate 3). The epithelium of the preserved tubules occasionally contained material that gave a positive Hale reaction and a positive alcian blue staining. In the epithelium of the collecting tubes, glycogen granules were still numerous. In the liver, the acino-peripheral lack of glycogen was still significant, and was generally combined with pronounced fatty infiltration of the parenchymal cells.

Serum changes in lethal DCA intoxication

Severe kidney damage that can clearly be demonstrated morphologically would be expected to cause uraemia. Changes in blood chemistry were followed for 7 days in rabbits exposed to a potentially lethal dose of 202 ppm for 1 hr. Three of the nine animals died 1 day after exposure, and after 8 days, three were killed for histological studies.

As expected, BUN rose rapidly and irreversibly (Fig. 1), and this was accompanied by a similar increase in inorganic phosphate and a correlating decrease in calcium values. The morphological changes were identical with those found in the animals exposed to 307 ppm for 1 hr. Kidney weight increased to 140% of that of the controls. The behaviour of the serum enzymes was striking (Fig. 1); SGOT increased fairly rapidly 1 day after exposure and returned to normal during the following days, while alkaline phosphatase increased only slightly on day 1 after exposure and decreased on subsequent days, and LDH decreased markedly until day 4, returning to normal thereafter. These changes in enzyme activities were indicative of acute but reversible liver-cell damage. Morphological parallels were seen in the reversible disappearance of glycogen, chromatolysis, fat infiltration and occasional necrosis following 1-hr exposure to 307 ppm DCA.

Morphological and serum changes in sublethal intoxications

To investigate the quantitative significance and time course of these renal lesions in the sublethal DCA intoxications likely to occur in man, a further ten rabbits were exposed to a sublethal concentration of 126 ppm DCA for 1 hr. A slight and transitory increase in BUN occurred only in some of the animals, and inorganic phosphate values increased slightly during days 3–4. After 9 days, all values had returned to normal. All marker enzymes for liver damage, especially the transaminases, fell within the normal range throughout.

Pathohistological changes included numerous calcified tubules visible in the kidney sections after day 14. In addition, there was a focal increase in connective tissue with formation of collagenous fibres (Plate 4a). The scarred areas were characterized by thickening of the basal lamina of the tubules and of Bowman's capsule. Many of the damaged tubules showed partial regeneration of the epithelium and, in some, cellular complexes protruded into the lumen. The liver parenchyma was partly rich in glycogen in some areas and showed a slight deposition of medium-sized or large lipid droplets.

After 4 wk, only some residues of calcification were found in the renal tubules, but scars were present in large areas of the cortex, combined with a pronounced thickening of the basement membranes of the tubules lying within the cortex. These tubules were strikingly basophilic. The liver parenchyma was characterized, at that period, partly by loss and partly by intensive storage of glycogen.

Scar formation in the kidney had progressed considerably by month 3 after exposure, but calcified tubules were rare. Especially striking were numerous tubules with markedly vacuolated epithelium (Plate 4b). Within the epithelial vacuoles neither fat, glycogen nor mucopolysaccharides could be demonstrated. After 3 months, the stucture of the liver parenchyma was normal, except in the peripheral regions of the lobules, in which a slight loss of glycogen was detected.

The relative weights of kidney, liver and lung are presented in Fig. 2. Kidney weight increased significantly 2 wk after exposure, probably because of an increase in the fluid content of the necrotic areas. In accordance with the shrinkage of scarred renal tissue, however, the kidney weight was below normal values by wk 12. The decrease in lung weight 4 and 12 wk after exposure was unexpected, because morphologically these organs appeared normal.

Sublethal exposure to DCA for 6 hr

Possible differences between the pattern of tissue reactions after a 1-hr exposure and that after more prolonged exposure were studied in ten rabbits that

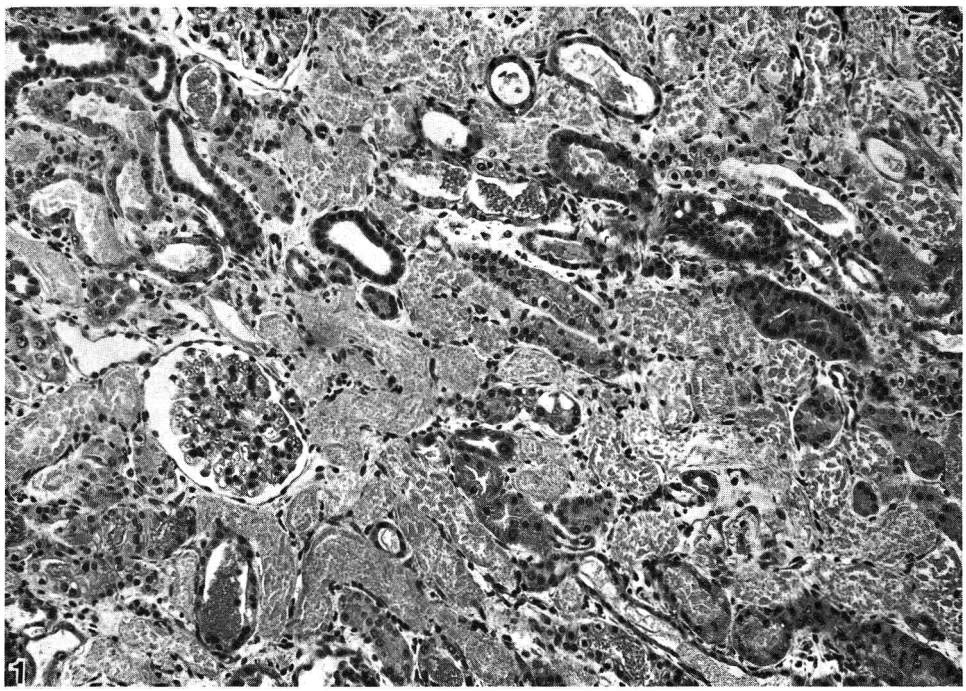

Plate 1. Extensive tubular necrosis in the rabbit kidney 48 hr after exposure to 307 ppm dichloroacetylene for 1 hr. Haematoxylin and eosin (H/E) × 240.

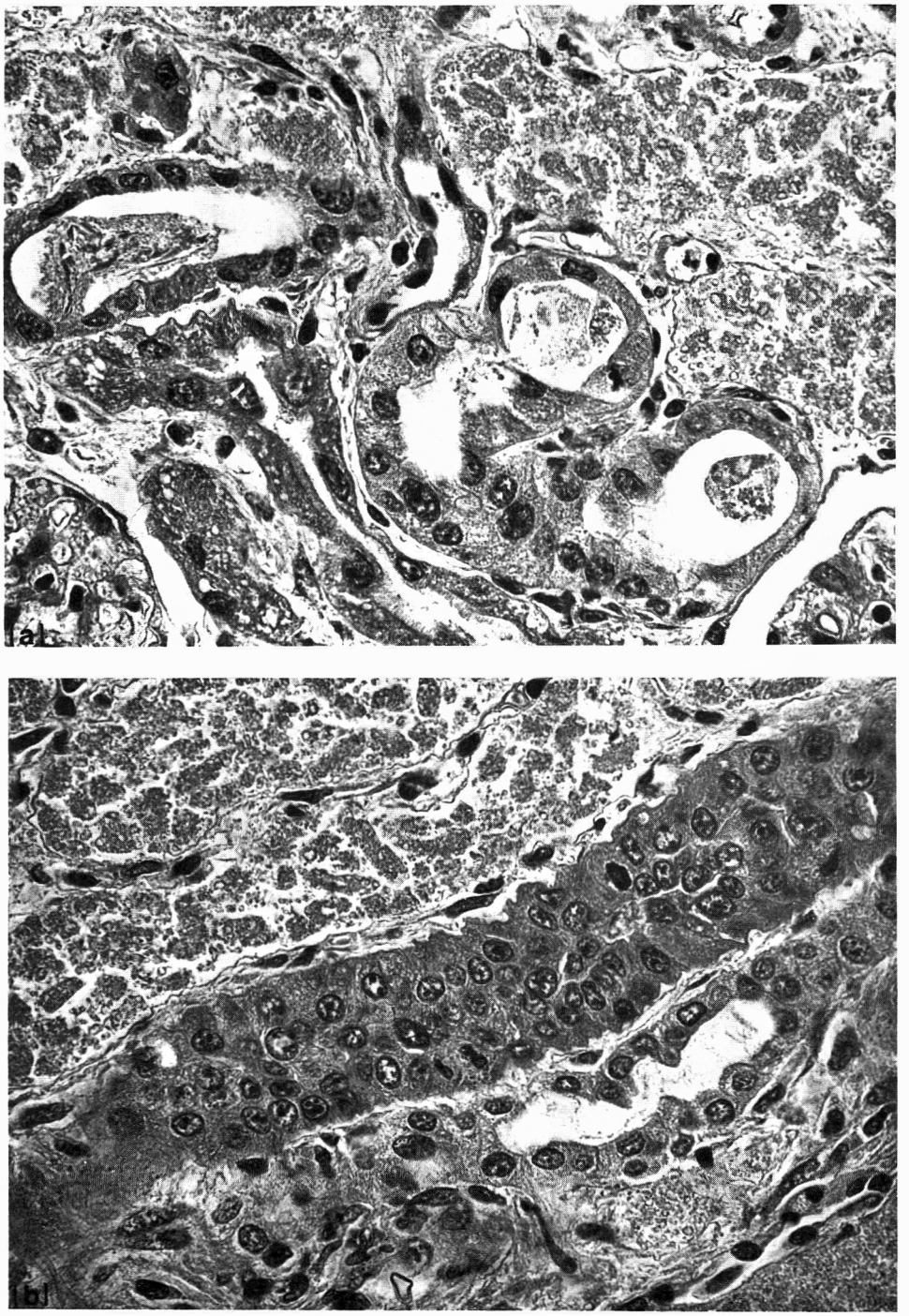

Plate 2. Tubular regeneration in sections of rat kidney 96 hr after exposure to 307 ppm dichloroacetylene for 1 hr, showing necrotic tubules at top right in (a) and top left in (b), a basophilic tubulus (centre) with abnormal mitoses and numerous strikingly large nuclei. An increase in cell number is apparent in (b). H/E × 560.

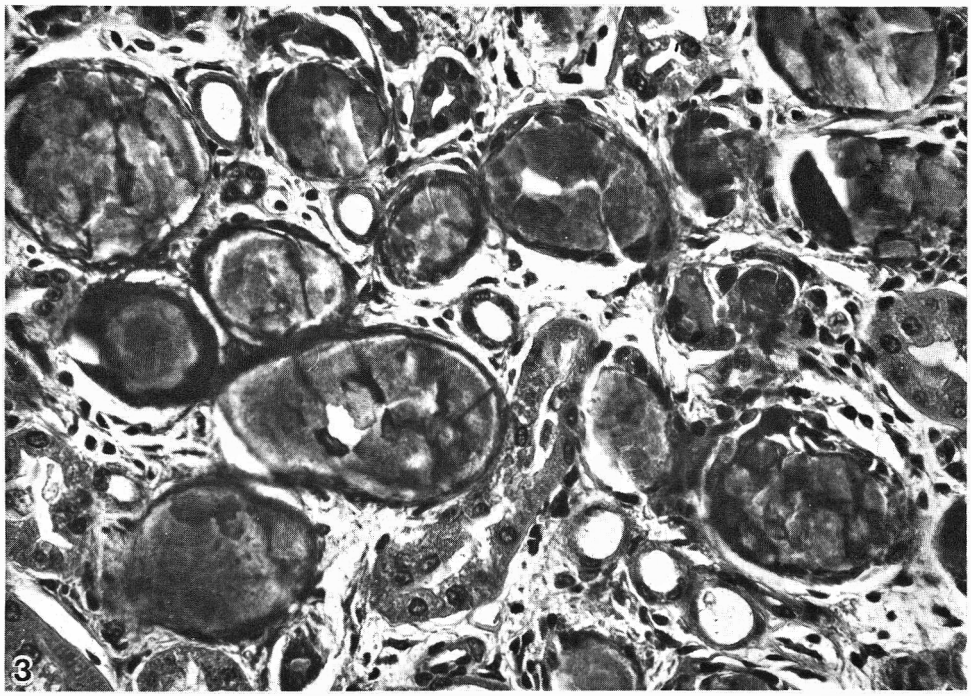

Plate 3. Numerous calcified tubules in the rabbit kidney 168 hr after exposure to 307 ppm dichloroacetylene for 1 hr. H/E × 350.

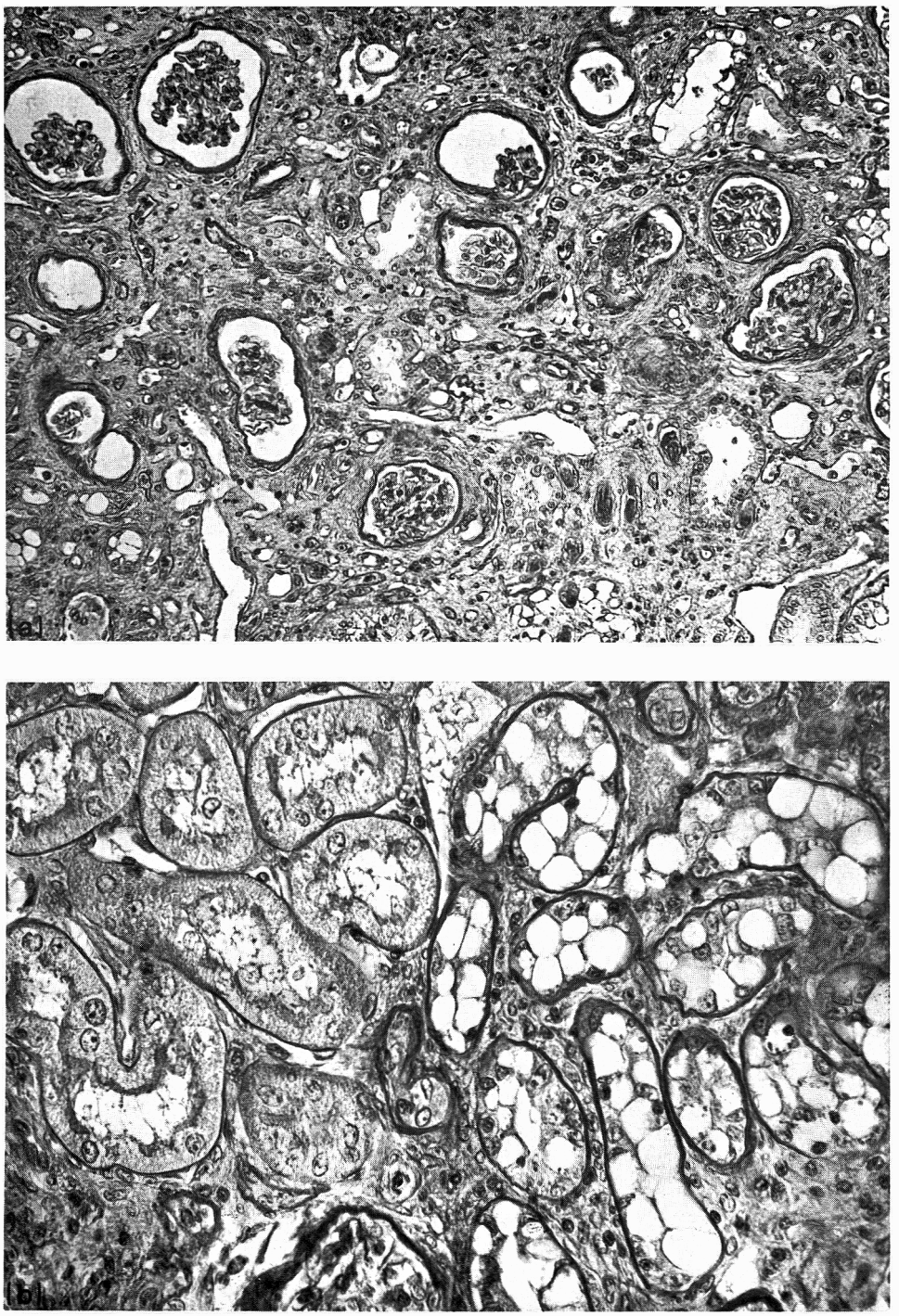

Plate 4. (a) Large scarred areas in the renal cortex of a rabbit 14 days after exposure to 126 ppm dichloroacetylene for 1 hr. Tri-PAS × 240. (b) Severe epithelial vacuolization and thickening of the basement membranes in the renal tubules of a rabbit 3 months after exposure to 126 ppm dichloroacetylene for 1 hr. Tri-PAS × 350.

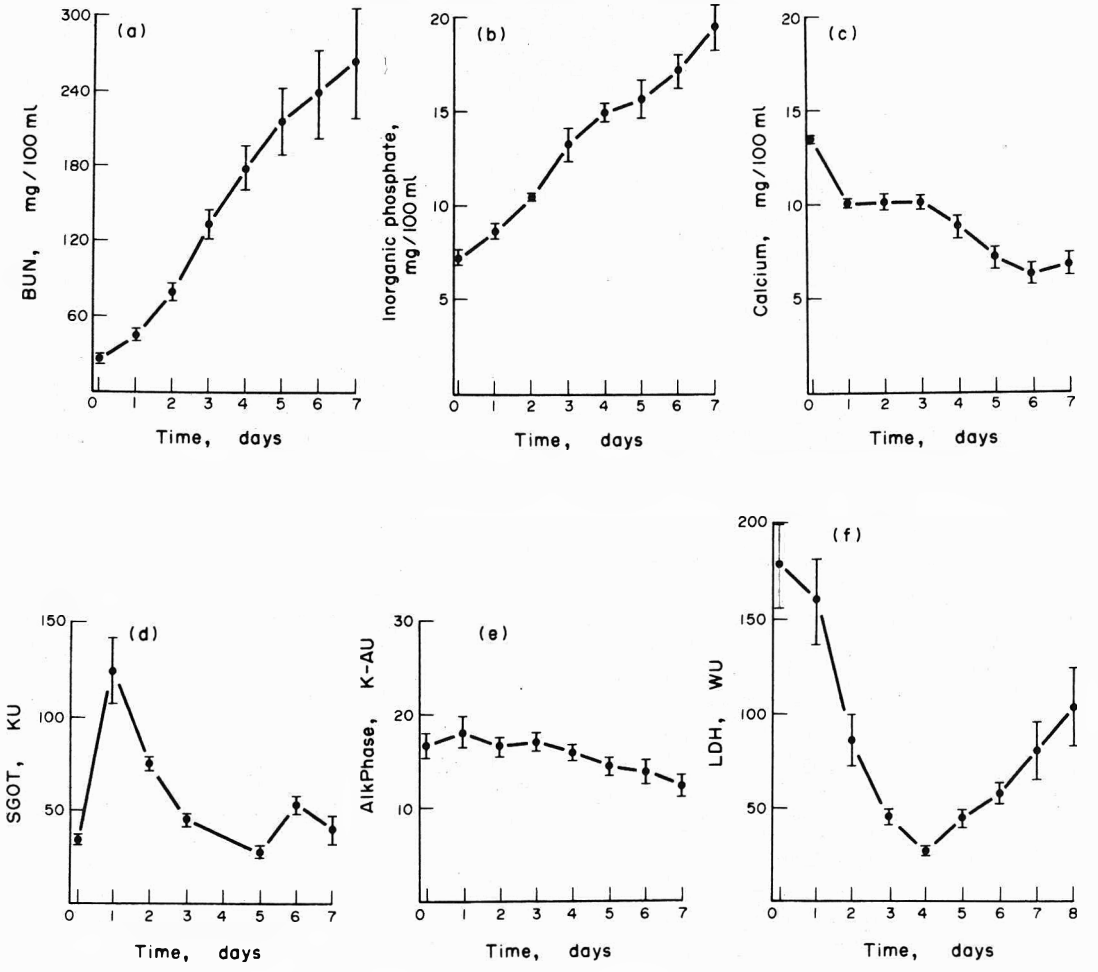

Fig. 1. Levels of (a) blood urea nitrogen, (b) inorganic phosphate, (c) calcium, (d) glutamic–oxalacetic transaminase, (e) alkaline phosphatase and (f) lactate dehydrogenase in the serum of rabbits exposed to 202 ppm dichloroacetylene for 1 hr. Values are means ± SD for six animals.

inhaled 17–23 ppm DCA for 6 hr. Four animals died 1 or 2 days after exposure. The BUN concentrations of the surviving animals (Fig. 3) revealed that the kidney damage was most pronounced 3 days after exposure, after which time the BUN concentrations decreased very slowly over 14 days, but without reaching normal values. This contrasted with the BUN profile after acute lethal intoxication, in which the kidney damage caused an irreversible BUN increase. The changes in serum transaminases were identical,

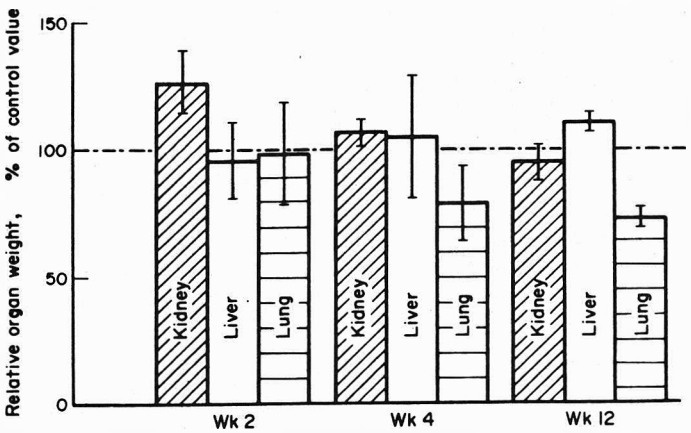

Fig. 2. Relative wet weights (g organ weight/kg body weight) of kidneys (■), liver (□) and lungs (▤) of rabbits, 2, 4 and 12 wk after exposure to 126 ppm dichloroacetylene. The relative weights (means ± SD for three animals), are expressed in each case as a percentage of the control value.

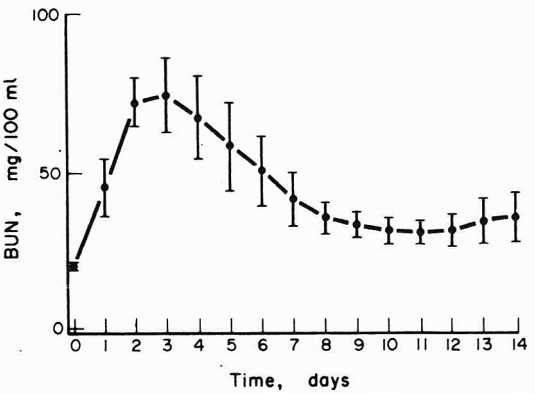

Fig. 3. Time-concentration curve for blood urea nitrogen in rabbits following exposure to 17–23 ppm dichloroacetylene for 6 hr. Values are means ± SD for six animals.

however, after sublethal and lethal intoxication, being limited in both cases to a transient increase during day 1 (Fig. 1).

Histological changes in the organs of these animals after 4 and 12 wk were identical to those found in animals exposed to 126 ppm DCA for 1 hr. Again, numerous intensely vacuolated cells were detected in the epithelium of the kidney tubules and the kidney weights were reduced (to 93% of control values).

DISCUSSION

The toxic effects of DCA differ from those of other C_1 and C_2 chlorinated hydrocarbons, in that the nephrotoxic potential of DCA markedly prevails over its hepatotoxicity. In the homologous series of chlorinated ethylenes, the hepatotoxic effect is always of major importance, and in the series of chlorinated alkanes, only chloroform and 1,1,1-trichloroethane are comparable in nephrotoxic activity (Plaa & Larson, 1965). A clear-cut correlation between the number and steric arrangement of chlorine atoms and the nephrotoxic potency of the compounds cannot, however, be demonstrated at present.

Furthermore, it must be emphasized that case reports of DCA poisoning do not provide any evidence of a predominantly nephrotoxic action in man, a point that has already been discussed in some detail (Reichert *et al.* 1975). The neurotoxicity, which is centred primarily on the cranial nerves, is the predominant symptom, and even in lethal intoxications the histological findings have not indicated a nephrotoxic effect comparable to that seen in experimental animals (Buxton & Hayward, 1967).

As used in animal experiments, for which it is stabilized with trichloroethylene, DCA does not induce any change in the alveolar structure of the lung. This surprising fact is in some contrast to the pronounced chemical reactivity of DCA; chlorine substitution of one hydrogen atom in the molecule of acetylene results in a striking increase in reactivity (Roedig, Bischoff, Heinrich & Märkl, 1963; Roedig & Detzer, 1971; Roedig, Detzer & Friedrich, 1964; Roedig, Märkl & Heinrich, 1963). Whilst acetylene incineration in the presence of oxygen is initiated only above 400°C, mono- and dichloroacetylenes decompose spontaneously at normal temperature when mixed with air. The reason for this striking difference in behaviour in the presence of oxygen may be the different degrees of stability of the peroxides formed by side-chain oxidation. Trichloroethylene apparently acts as an inhibitor of peroxide formation. Whether and to what extent a nucleophilic attack at the site of the C–Cl bond after uptake into the organism may be influenced by the inhibitor is worthy of investigation in comparative experiments with other compounds that exert some stabilizing effect (such as acetylene, ethylene and its chlorinated homologues).

Most of the toxic lesions observed in the kidney and liver were non-specific. A loss of glycogen with accompanying chromatolysis predominantly in the acino-central parts of the liver lobules has been described after intoxication with various compounds (Bannasch, 1975a, b). In all these intoxications, the cytoplasmic changes were closely related in time and location to liver-cell necrosis, but their significance in the development of necrosis has not yet been fully elucidated (Bannasch, 1975b; Magee, 1966).

Renal tubular necrosis is a common consequence of acute intoxications (Zollinger, 1966), particularly after the intake of heavy metals like mercury or uranium. In these cases, secondary calcification of the necrosis has already been described (Zollinger, 1966). Tubular necrosis induced by DCA inhalation results in uraemia, which kills many animals. After the acute phase of intoxication, the renal histology is characterized by tubular regeneration and scarring. The mitoses observed during the regeneration process are often atypical. Since these anomalous mitoses may result in the formation of polyploid macronuclei (Altmann & Müller, 1973), they may also be responsible for the enlargement of numerous nuclei of the tubular epithelium previously described by Jackson *et al.* (1971) in DCA-exposed animals. The genesis of the vacuoles that appear in the tubular epithelium at later stages still remains to be elucidated; in our experiments we were not able to demonstrate any storage of fat, glycogen or mucopolysaccharides inside the vacuoles.

It should be emphasized that the livers of some DCA-treated animals show a striking increase in glycogen content and that in some renal tubules an accumulation of polysaccharides can be demonstrated by histochemical methods. Since similar changes have been found in a variety of other experimental models during the early stages of the development of liver and kidney tumours (Bannasch, 1975b), the testing of DCA for carcinogenic potential seems a worthwhile study.

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SHORT-TERM TOXICITY STUDY OF ZINC DIBUTYLDITHIOCARBAMATE IN RATS

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Abstract—Groups of 15 male and 15 female rats were given diet containing 0 (control), 100, 500 or 2500 ppm zinc dibutyldithiocarbamate (ZnDBDTC) for 17 wk. At the highest treatment level, the rate of body-weight gain of the females and the food intake of both sexes were reduced. There were no effects attributable to ZnDBDTC in the results of the haematological examination or the analyses of serum and urine. The relative weights of the liver and kidneys were increased in rats of both sexes given 2500 ppm ZnDBDTC. There were no differences between treated and control rats in the histological appearance of any of the tissues examined. The no-untoward-effect level was 500 ppm in the diet, providing an intake between 41 and 47 mg ZnDBDTC/kg/day.

INTRODUCTION

Zinc dibutyldithiocarbamate (ZnDBDTC) is a vulcanization accelerator for natural rubber and latex and a stabilizer for rubber-based adhesive systems, isobutylene–isoprene copolymers and polypropylene. It is used in a number of rubber and rubber-based materials for food packaging and food handling, e.g. conveyor belts.

At present there are no specific regulations governing the use of additives in packaging materials in the UK. ZnDBDTC was included by the Council of Europe in a preliminary classification of substances for use in packaging materials for food contact, but additional data related to safety were considered to be necessary (Ministry of Agriculture, Fisheries and Food, 1976). In the USA, ZnDBDTC is permitted for use in can-end cements, adhesives for food packaging, sealing gaskets for food containers and rubber articles intended for repeated use in contact with food (Code of Federal Regulations, Secs 121.2514, .2520, .2550 and .2562, respectively).

A 90-day study, involving oral administration of ZnDBDTC to five rabbits at a dose level of 50 mg/kg/day, was carried out by F. B. Flinn (unpublished report 1938). No histological changes were observed and the erythrocyte and leucocyte counts were unaffected. In a more recent study, ZnDBDTC was given to mice at the maximum tolerated level (2600 ppm in the diet) for 18 months. The compound was administered initially by oral intubation at a dose level of 1000 mg/kg/day from day 7 to day 28 of age and thereafter in the diet at a level of 2600 ppm. No evidence of carcinogenicity was observed (Innes, Ulland, Valerio, Petrucelli, Fishbein, Hart, Pallotta, Bates, Falk, Gart, Klein, Mitchell & Peters, 1969). There appear to be no other published data relating either to the toxicological properties of ZnDBDTC or to its metabolic fate. However, the toxicology of certain related dithiocarbamates, in particular of the pesticide zinc dimethyldithiocarbamate, has been reviewed by Engst & Schnaak (1974), and Eisenbrand, Ungerer & Preussmann (1974) have reported the formation of nitrosamines in the rat stomach after administration of nitrite and dialkyldithiocarbamates.

The present paper describes a short-term safety evaluation study carried out in rats fed ZnDBDTC in the diet.

EXPERIMENTAL

Materials. The sample of ZnDBDTC used in these studies was supplied by W. R. Grace Ltd., St. Neots, Cambs., and complied with the following specification: A soft white, free-flowing, crystalline powder with a mild odour characteristic of thiocarbamates and no odour of amines or sulphur; melting range, 103–112°C; ash, 20 ± 3%; zinc content, 13·0–15·0%; total sulphur, 26·0–30·0%; chloroform insolubles, max 4·0%; infra-red spectrum matching published spectrum of standard material.

Animals and diet. Rats of a Wistar strain were obtained from a specified-pathogen-free colony (A. Tuck & Son, Rayleigh, Essex) and housed five in a cage in an animal room maintained at 21° ± 1°C and 50–70% relative humidity. They were given ground Spillers' Laboratory Small Animal Diet and water *ad lib.*

Experimental design and conduct. Groups of 15 male and 15 female rats were given diets containing 0 (control), 100, 500 or 2500 ppm ZnDBDTC for 17 wk. Additional groups of five rats of each sex and of similar body weight were given diets containing 0, 500 or 2500 ppm ZnDBDTC for 2 or 6 wk. The animals were weighed initially, on days 1, 2, 6, 9, 13, 16 and 20 of treatment and thereafter at weekly intervals up to wk 17. Food and water consumptions were measured over a 24-hr period preceding each weighing.

Urine was collected from each rat during the last week of its treatment and examined for appearance, microscopic constituents and content of albumin, glu-

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cose, ketones, bile salts and blood. At the same times, renal concentrating and diluting ability was assessed by measuring the specific gravity and volume of the urine produced during a 6-hr period of water deprivation and in a 2-hr period following a water load of 25 ml/kg. In addition, at wk 6 and 17, the same measurements were made on the urine produced during a 4-hr period following 16 hr without water. The number of cells in the urine was counted using the 2-hr sample.

At the end of the appropriate period of feeding the rats were killed by exsanguination from the aorta under barbiturate anaesthesia following a 24-hr period without food. The blood obtained was examined for haemoglobin concentration, packed cell volume and counts of erythrocytes and total and differential leucocytes. Slides were prepared from all blood samples for the counting of reticulocytes, but these counts were confined to the control rats and the rats given the highest dietary level of ZnDBDTC. Serum was analysed for the activities of glutamic–oxalacetic transaminase, glutamic–pyruvic transaminase and lactate dehydrogenase and for its content of urea and glucose.

An autopsy was carried out on each animal, during which any macroscopic abnormalities were noted and the brain, heart, liver, stomach, small intestine, caecum, spleen, kidneys, adrenal glands, gonads, pituitary and thyroid were weighed. Samples of these organs and of lung, salivary gland, trachea, oesophagus, aorta, thymus, various lymph nodes, urinary bladder, colon, rectum, pancreas, uterus, spinal cord and skeletal muscle were preserved in 10% buffered formalin. Paraffin-wax sections of all of these tissues were stained with haematoxylin and eosin for microscopic examination, but the examination was performed only on the tissues from all the rats given 2500 ppm ZnDBDTC for 17 wk and half of the control rats, on the liver, kidneys, spleen, heart and lungs from half of the rats fed 100 or 500 ppm ZnDBDTC for 17 wk and on all the tissues from animals in which abnormalities were seen at autopsy.

RESULTS

The mean body weights of the female rats given diet containing 2500 ppm ZnDBDTC were lower than those of the controls and the differences were statistically significant from day 9 onwards (Table 1). The mean body weights of the male rats receiving 500 or 2500 ppm ZnDBDTC were also slightly lower than those of the controls but to a statistically significant degree only in the 500 ppm group on days, 13, 16, 20 and 27. At the dietary level of 100 ppm ZnDBDTC the mean body weights of both the male and the female rats were consistently, although not significantly, greater than those of the control rats. In both sexes given 2500 ppm ZnDBDTC, the overall mean food consumption was significantly lower than the corresponding control value (Table 1). There were no statistically significant differences between the treated and the control rats with respect to water consumption. The mean calculated daily intakes of ZnDBDTC over the test period were 9, 41 and 202 mg/kg for the male groups given dietary levels of 100, 500 and 2500 ppm, respectively, and 9, 47 and 228 mg/kg for the corresponding females.

The haemoglobin concentration was significantly higher than the control value at wk 2 in the male rats fed 500 ppm and in the female rats fed 2500 ppm ZnDBDTC (Table 2). In the latter, the packed cell volume was also higher, although not to a statistically significant extent. There were no such changes at subsequent examinations with the exception of a slightly higher value for packed cell volume in the female rats given 2500 ppm ZnDBDTC for 17 wk. At wk 6 the proportion of neutrophils was significantly greater and the proportion of lymphocytes significantly lower than the control values in the female rats given 2500 ppm ZnDBDTC, while at wk 17 these changes were reversed. In the results of serum analyses, statistically significant differences ($P < 0.05$) between treated and control rats were limited to a rise in glutamic–pyruvic transaminase and a fall in lactate dehydrogenase at wk 6 in males on the highest dietary level.

Table 1. *Mean body weights and food intake of rats fed diets containing 0–2500 ppm ZnDBDTC for up to 17 wk*

Dietary level (ppm)	Body weight (g) at day						Food intake (g/rat/day) at day						Mean food intake (g/rat/day)
	0†	2	27	55	83	120	0†	2	27	55	83	120	
Males													
0	77	92	248	346	404	450	11·2	13·2	20·3	21·6	20·7	20·2	19·9
100	76	90	254	360	421	477	10·5	12·1	21·7	25·3	20·5	21·7	20·7
500	77	89	233*	331	389	439	10·9	10·9	20·7	22·0	20·1	20·7	19·2
2500	77	89	236	325	373	425	11·1	9·7	20·4	21·3	17·3	19·9	18·7*
Females													
0	72	83	173	216	242	261	10·7	11·4	15·7	18·1	15·8	17·3	16·0
100	74	86	178	221	249	267	10·5	11·1	16·2	17·5	15·7	15·0	15·6
500	73	83	164	213	239	258	10·6	10·1	16·1	18·0	16·5	17·3	16·0
2500	72	82	157**	193**	213***	227***	12·0	10·3	14·4	15·3	14·5	14·3	14·3*

ZnDBDTC = Zinc dibutyldithiocarbamate

†First day of treatment.

Values of body weights are the means for groups of 15 animals. Values for food consumption are the means for three cages of five animals. Figures marked with asterisks differ significantly from those of the appropriate controls (White (1952) for the mean food intake and the Student's t test for body weight): *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

Table 2. *Haematological values for rats fed diets containing 0–2500 ppm ZnDBDTC for 2, 6 or 17 wk*

Sex and dietary level (ppm)	No. of rats	Hb (g/100 ml)	PCV (%)	RBC (10^6/mm^3)	Retics (% of RBC)	Leucocytes				
						Total (10^3/mm^3)	Differential (%)			
							N	E	L	M
Wk 2										
Male										
0	5	13·4	41	6·27	3·1	4·0	11	1	88	0
500	5	14·7*	42	6·62	—	5·0	7	0	93	0
2500	5	14·4	42	6·74	3·8	3·8	14	0	86	0
Female										
0	5	14·6	42	6·76	1·4	3·3	17	0	83	0
500	5	15·8	44	6·96	—	3·5	11	0	89	0
2500	5	16·9**	45	7·60	1·5	4·2	16	0	84	0
Wk 6										
Male										
0	5	15·7	46	7·69	1·9	6·1	6	1	93	0
500	5	16·3	46	7·92	—	4·6	4	1	96	0
2500	5	16·6	47	7·97	1·3	6·4	10	0	90	0
Female										
0	5	14·5	41	7·52	1·1	3·5	6	0	93	1
500	5	14·6	42	7·49	—	3·0	8	0	92	0
2500	5	14·2	41	7·97	1·2	2·5	15*	0	84*	1
Wk 17										
Male										
0	15	15·4	44	7·51	1·5	4·3	23	1	74	2
100	15	15·7	43	7·58	—	4·1	22	1	77	0
500	15	15·8	44	7·60	—	4·0	21	1	78	0
2500	15	15·7	44	7·35	1·8	3·9	20	1	77	2
Female										
0	15	15·4	44	7·27	1·6	3·1	19	1	78	2
100	15	15·3	44	6·91	—	3·0	21	1	78	0
500	15	15·6	44	7·08	—	3·0	25	1	73	1
2500	15	15·8	45*	6·78	1·5	3·4	14**	1	83**	2

ZnDBDTC = Zinc dibutyldithiocarbamate

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

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

Basophils did not account for more than 0·5% of the leucocytes in any group and inclusions were not seen in the erythrocytes.

A dash indicates that the count was not carried out.

No abnormal urinary constituents were found in any of the animals and the urinary protein levels were similar in treated and control rats. There were scattered significant differences between treated and control rats in the volume and specific gravity of the urine in the dilution test at wk 2 and 6 but not at wk 17 (Table 3). The number of cells in the urine tended to be lower in the treated than in the control rats and in some instances these differences were statistically significant.

There were a number of small but statistically significant differences between the treated and control rats in the organ weights and in organ weights expressed relative to body weight (Table 4). At wk 2 the heart weight and the relative heart weight of the female rats fed 2500 ppm ZnDBDTC were significantly lower than control values, the relative caecum weight was significantly higher in both sexes given 2500 ppm and in the males fed 500 ppm and the relative liver weight in rats of both sexes given 2500 ppm was slightly higher than the control value, although this difference was statistically significant only in the males. At wk 6 the brain weight of the male rats fed 2500 ppm ZnDBDTC was significantly lower ($P < 0.05$) than that of the controls and there were small decreases in the weights of several other organs. In terms of relative organ weights, these differences were not evident but the relative weights of the caecum and gonads showed significant ($P < 0.05$) increases in these males. At wk 17 the relative weights of the kidney and liver were significantly higher than the control values in both sexes of rats receiving 2500 ppm ZnDBDTC. In the male rats at this dietary level the relative weights of the small intestine and caecum were also higher. The weights of the brain, heart, small intestine and caecum in the female rats given 2500 ppm ZnDBDTC were significantly lower than the corresponding control values. However, the relative heart weight was significantly increased as was the relative weight of the adrenals in the females given 500 or 2500 ppm ZnDBDTC. There were several other small changes in organ weights but these showed no relation either to the dietary level of ZnDBDTC or the duration of treatment.

Table 3. *Mean values of renal concentration/dilution tests and urinary cell excretion in rats fed diets containing 0–2500 ppm ZnDBDTC for 2, 6 and 17 wk*

Sex and dietary level (ppm)	No. of rats	Cell excretion (10^3/hr)	Concentration test: Specific gravity 0–6 hr	Concentration test: Specific gravity 16–20 hr	Concentration test: Volume (ml) 0–6 hr	Concentration test: Volume (ml) 16–20 hr	Dilution test (2 hr): Specific gravity	Dilution test (2 hr): Volume (ml)
Wk 2								
Male								
0	5	3·0	1·049	—	1·2	—	1·008	2·9
500	5	2·4	—	—	1·1	—	1·008	4·5
2500	5	1·8	—	—	1·4	—	1·009	3·5
Female								
0	5	5·0	1·063	—	1·3	—	1·015	3·9
500	5	2·5	1·052	—	1·0	—	1·005*	2·9
2500	5	2·8	1·053	—	0·9	—	1·006*	3·4
Wk 6								
Male								
0	5	8·0	1·060	1·081	2·0	0·3	1·006	6·2
500	5	9·2	—	1·064	3·6	0·4	1·006	6·9
2500	5	5·0	1·071	1·078	1·8	0·2	1·009*	3·0*
Female								
0	5	6·3	1·072	1·087	0·6	0·2	1·007	3·8
500	5	1·4*	1·068	1·084	0·9	0·3	1·008	3·6
2500	5	3·2	1·076	1·078	1·2	0·2	1·007	2·8
Wk 17								
Male								
0	15	6·7	1·040	1·072	2·8	0·3	1·005	9·2
100	15	4·7	1·039	1·073	2·6	0·3	1·005	7·1
500	15	8·1	1·045	1·079	2·9	0·4	1·005	9·4
2500	15	3.2**	1·036	1·075	3·1	0·2	1·005	9·4
Female								
0	15	4·0	1·065	1·084	0·8	0·1	1·008	3·9
100	15	5·4	1·058	1·077	1·0	0·2	1·006	4·2
500	15	3·9	1·057	1·071	1·2	0·1	1·006	4·4
2500	15	3·7	1·057	1·081	0·7	0·1	1·006	4·2

ZnDBDTC = Zinc dibutyldithiocarbamate

Results are means for the numbers of rats shown, and those marked with an asterisk differ significantly (ranking method of White, 1952) from those of controls: $*P < 0.05$; $**P < 0.01$.

Tests for glucose, bile salts, blood and ketones were negative.

A dash indicates that the measurement was not carried out.

No histological changes related to the duration or level of treatment were seen in any of the tissues examined.

DISCUSSION

The decrease in the rate of body-weight gain of the female rats fed 2500 ppm ZnDBDTC was paralleled by a reduction in the mean food intake, as was the less marked reduction of body weight in the males. In this case the reduction in food intake was maximal during the first few days of the study, a pattern often associated with an unpalatable diet.

The increased haemoglobin concentrations in both sexes of treated rats at wk 2 were accompanied by small increases in the packed cell volume and erythrocyte count. However, there were no statistically significant changes in any of these parameters at subsequent intervals, with the exception of a 2·0% increase in the packed cell volume of the female rats fed 2500 ppm ZnDBDTC for 17 wk, and it is unlikely that these findings on the erythrocytes represented an effect of treatment. The statistically significant alterations in the ratio of the different types of leucocytes in the female rats given 2500 ppm ZnDBDTC were in opposite directions at 6 and 17 wk and there were no such changes in the male rats. These considerations suggest that, like the erythrocyte differences, these leucocyte changes were due to chance rather than to an effect of treatment.

The isolated differences in the serum analyses and the renal function tests were not consistent in direction, not matched by the results for the corresponding groups of rats of the opposite sex and showed no relationship to the level or duration of treatment. The findings were not indicative of an adverse effect and are not considered to be related to ZnDBDTC treatment.

Most of the differences in organ weights occurred in the animals that had failed to gain weight at a rate comparable with the controls. In general these differences consisted of lower weights which, when expressed relative to body weight, were normal or increased. This pattern has been seen repeatedly in these laboratories (Carpanini, Gaunt, Hardy, Gangolli, Butterworth & Lloyd, 1978; Gaunt, Sharratt,

Table 4. *Relative organ weights of rats fed diets containing 0–2500 ppm ZnDBDTC for 2 or 17 wk*

Sex and dietary level (ppm)	No. of rats	Relative organ weights (g/100 g body weight)												Terminal body weight (g)
		Brain	Heart	Liver	Spleen	Kidneys	Stomach	Small intestine	Caecum	Adrenals†	Gonads‡	Pituitary†	Thyroid†	
Wk 2														
Male														
0	5	0·99	0·44	3·80	0·47	0·88	0·70	4·20	0·50	36·9	1·13	4·1	10·9	155
500	5	1·12	0·47	3·71	0·47	0·89	0·85*	4·21	0·62*	34·9	1·34	5·1**	11·5	141
2500	5	1·09	0·44	4·21**	0·52	0·94	0·73	4·15	0·69***	37·4	1·30	4·4	10·5	144
Female														
0	5	1·02	0·42	3·58	0·36	0·92	0·71	4·25	0·50	60·3	84	4·8	11·1	132
500	5	1·05	0·43	3·87	0·39	0·94	0·74	4·07	0·57	42·2**	81	5·3	17·3*	128
2500	5	1·12	0·38*	3·88	0·34	0·95	0·73	3·80	0·59*	47·1	84	4·6	16·8	126
Wk 17														
Male														
0	15	0·44	0·25	2·44	0·17	0·54	0·37	1·75	0·26	15·3	0·80	1·9	4·4	432
100	15	0·42	0·25	2·45	0·17	0·53	0·38	1·77	0·27	16·6	0·76	2·2	3·8	457
500	15	0·44	0·25	2·50	0·19	0·55	0·39	1·84	0·29	13·7	0·81	2·2	4·3	423
2500	15	0·45	0·27	2·69**	0·19	0·62**	0·40	1·89*	0·30*	15·3	0·86	2·2	4·9	403
Female														
0	15	0·71	0·31	2·38	0·21	0·59	0·52	2·36	0·38	28·5	49	4·5	7·1	254
100	15	0·70	0·30	2·34	0·22	0·58	0·51	2·34	0·35	31·1	50	4·6	7·0	259
500	15	0·72	0·31	2·40	0·20	0·59	0·50	2·41	0·34*	33·0*	51	4·7	6·9	248
2500	15	0·77	0·33*	2·59***	0·22	0·64**	0·56	2·50	0·37	33·5*	54	4·5	7·4	219***

ZnDBDTC = Zinc dibutyldithiocarbamate

†Weights of this organ are expressed in mg/100 g body weight.
‡Weights of female gonads are expressed in mg/100 g body weight.
Figures are means for the numbers of rats shown. Those marked with asterisks differ significantly (Student's t test) from the control value: *$P < 0·05$; **$P < 0·01$; ***$P < 0·001$.

Grasso, Lansdown & Gangolli, 1974; Gray, Butterworth, Gaunt, Grasso & Gangolli, 1977) and in animals with low body weights due to a reduced intake of normal food (Feron, de Groot, Spanjers & Til, 1973; Peters & Boyd, 1966; Schwartz, Tornaben & Boxhill, 1973), and it is therefore more likely that the differences were due to the indirect effects of a reduced food intake than to a toxic effect.

The relative caecal weights of the treated animals were higher than those of the controls in the early stages of the study. The toxicological significance of such caecal enlargement remains a matter of debate (Butterworth, Lake, Mason & Rowland, 1975), but increases in the relative weight of the caecum have been observed in rats fed restricted amounts of food (Peters & Boyd, 1966) and the increased caecal weight of the ZnDBDTC-treated rats may reflect their initially decreased food intake. That it does not reflect a direct and progressive effect of ZnDBDTC is suggested by the fact that at wk 17 there was very little difference in relative caecum weight between the treated and control rats of either sex.

The relative weights of the kidneys and liver were significantly increased in rats of both sexes given 2500 ppm ZnDBDTC for 17 wk. In neither organ were there any histopathological changes and in the case of the kidneys, the renal function tests provided no evidence of any functional impairment. An increase in liver weight unaccompanied by histological changes is frequently observed in experimental animals treated with high levels of foreign compounds (Barka & Popper, 1967). Such increases, when accompanied by increased levels of the hepatic microsomal drug-metabolising enzymes, have been considered as an adaptive physiological response to increased metabolic demand (Crampton, Gray, Grasso & Parke, 1977; Gray, 1975; Schulte-Hermann, 1974). In the absence of additional biochemical data, however, it is not possible to determine whether the increase in liver weight elicited by ZnDBDTC can be explained in this manner.

In view of the increases in kidney and liver weight at the highest dietary level of ZnDBDTC, it is concluded that the no-untoward-effect level in this study is 500 ppm in the diet. This provided overall intakes of 41 and 47 mg/kg/day for the male and female rats, respectively, intakes which, after application of a 100-fold safety factor, would correspond to an acceptable daily intake for a 70-kg adult in the range of 29–33 mg. However, for these amounts to be related to the possible likely human hazard from exposure to ZnDBDTC from food-packaging materials, information on the extent to which ZnDBDTC migrates from packaging into food under various storage conditions will be required.

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LONG-TERM TESTING OF PATULIN ADMINISTERED ORALLY TO SPRAGUE–DAWLEY RATS AND SWISS MICE

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Abstract—Over a period of 64 wk, a series of twice weekly doses totalling 358 mg patulin/kg were administered by stomach tube to 50 female Sprague–Dawley rats from weaning, to investigate the possible carcinogenic action of the mycotoxin. These animals and an appropriate control group were observed throughout their natural lifespan. In spite of a high spontaneous incidence of benign tumours in this Sprague–Dawley strain, no carcinogenic action attributable to the patulin administration was observed. Twelve pregnant Swiss mice received, by gastric intubation, twice-daily doses of 2 mg patulin/kg on days 14–19 of pregnancy, giving a total dosage of 24 mg/kg, to study the possible transplacental carcinogenicity of patulin. The offspring of these mice showed no evidence of such activity at the dose level used, but during the neonatal period (2–6 days of parturition) eleven of the 52 females and eight of the 43 males born to the patulin-treated dams died, all showing similar signs of toxicity, whereas no deaths occurred in the offspring of the control group within 6 wk of parturition.

INTRODUCTION

Patulin (also known as clavatin, claviformin, clavacin, expansin, mycosin, penicidin, tercinin, gigantin, leucopin and penatin) is a water-soluble β-unsaturated lactone, 4-hydroxy-4*H*-furo[3,2-*c*]pyran-2(6*H*)-one. It has been isolated from several species of Penicillium and Aspergillus, was found in 1944 to be effective, in a concentration of 1:20,000, as a nose and throat spray for the treatment of head colds, and can be inactivated by sulphur dioxide and by compounds bearing thiol groups (Ciegler, Detroy & Lillehoi, 1971).

In addition to its action against Gram-negative and Gram-positive bacteria, actinomycetes and protozoa, it has shown some carcinogenic potential. When 0·2 mg patulin was applied to rats twice weekly at the same site for 60 wk, local sarcomas were found in six of the eight rats that survived for 1 yr (Dickens & Jones, 1963). Ingestion of patulin by mice or rats induced no carcinogenic action (Dickens, 1967; Enomoto & Saito, 1973), but the authors did not report the number of animals used, the dosage or the duration of administration.

Rejected long ago as a chemotherapeutic agent, patulin has evoked interest more recently as a contaminant of certain foods and feedstuffs (Frank, 1970). In studies of the occurrence of this mycotoxin in different varieties of apples and pears with brown rot, patulin was found in about 50% of the samples investigated (Frank, Orth & Hermann, 1976; Harwig, Chen, Kennedy & Scott, 1973). Patulin levels as high as 1000 ppm were found in naturally rotten apples by Brian, Elson & Lowe (1956) and Frank *et al.* (1976). Significant diffusion into the healthy plant tissue was not observed in apples, but was demonstrated in pears, peaches and tomatoes. A natural patulin content has been found in apples, pears, peaches, apricots, bananas, pineapples and grapes. After inoculation with *Penicillium expansum*, *P. urticae* or *Byssochlamys nivea*, greengages, strawberries, honeydew melons, red and green paprika, tomatoes, cucumbers and carrots were found to contain patulin (Frank, Orth & Figge, 1977; Frank *et al.* 1976). In the making of apple puree under domestic conditions, the patulin content of the initial material is reduced by about 90%, but the heating of apple juice at 90°C for 10 min does not inactivate the mycotoxin (Drilleau, Bohuon & Dupuy, 1973), although it is rapidly inactivated in the presence of sulphur dioxide (Pohland & Allen, 1970).

A proposal to use *P. expansum*, a patulin-producing fungus, as a starter culture for fermented sausages was rejected on account of the liver lesions found in ducks treated orally with 0·2 mg patulin for 6 wk (Mintzlaff & Leistner, 1971). Consequently it was of interest to test patulin orally in a long-term experiment in rats and to investigate any possible transplacental action of patulin given orally to mice during the last third of pregnancy.

EXPERIMENTAL

Test material. Patulin, isolated by one of us (H.K.F.) from a liquid nutrient medium fermented with *P. expansum* and purified and crystallized according to Norstadt & McCalla (1969), was shown to be 98% pure by gas chromatography, thin-layer chromatography and infra-red spectroscopy. For administration to animals, it was dissolved in distilled water containing 0·05% lactic acid to avoid the possibility of partial decomposition of the patulin owing to its erratic stability at pH values above 7·0. The patulin solutions were prepared immediately before oral administration by stomach tube, the concentrations being adjusted to give a dosage volume of 1 ml/kg body weight for the rats and 0·1 ml/10 g body

weight for the mice. Control animals were given the appropriate volume of distilled water containing 0·05% lactic acid.

Animals and treatment

Rat study. Patulin was tested orally by long-term administration to groups of 50 random-bred specified-pathogen-free female Sprague–Dawley rats (Broekman Instituut, Helmond, The Netherlands), weighing initially 40 ± 3 g. The rats were housed in Makrolon cages (type II) and received Altromin R pellet diet (Altromin GmbH, Lippe) and water *ad lib.* Patulin was administered by stomach tube twice weekly (at intervals of 72 hr), the doses being 2 × 1 mg/kg weekly during wk 1–4 and 2 × 2·5 mg/kg weekly for the following 70 wk, to give a total dosage of 358 mg patulin/kg.

Mouse study. Sixty random-bred specified-pathogen-free female Swiss mice (Broekman Instituut) weighing 28 ± 1 g were housed together for 24 hr with males of the same strain for mating. Because it was not possible to confirm insemination of female mice by the presence of sperm in the vaginal smear, selection of 24 mice with distinct signs of pregnancy was carried out 14 days after mating. Of these mice, 12 received 2 × 2 mg patulin/kg daily by gastric intubation on the next 6 days, while the 12 controls were given the patulin-free solution according to the same schedule. Each of the pregnant mice was housed in a Makrolon cage type I, for control of the offspring. At weaning, the offspring were separated by sex and kept in groups of ten in Makrolon cages type II. Both dams and weanlings were given Altromin M pellet diet (Altromin GmbH) and water *ad lib.* The male offspring were castrated under ether narcosis when 6 wk old to avoid early deaths due to the aggressive behaviour of normal male mice. All animals were observed throughout life, and at death were autopsied and examined histologically.

RESULTS

Rat study

The patulin treatment had no effect on the weight gain of the rats and there was no significant difference between the average survival time of the treated and control groups (Table 1), since two of the patulin-treated rats died within the first 6 wk from pulmonary oedema resulting from intubation injuries. The numbers of tumour-bearing rats in the control and patulin-treated groups (42 and 37) and the numbers with malignant tumours (13 and 12) or with benign tumours provided no evidence that the total dosage of patulin administered had any carcinogenic action. Several rats developed two or three different types of tumour and consequently the numbers listed for specific types of malignant and benign tumours (Table 1) do not correspond with the total numbers of tumour-bearing rats.

The incidence of malignant tumours in both groups indicated no significant preponderance of any specific type of neoplasm. In the case of benign tumours, a

Table 1. *Survival and tumour incidence in female Sprague–Dawley rats treated in total with 358 mg patulin/kg given in twice-weekly doses over a 64-wk period*

Type of tumour	Tumour incidence* in Control group	Tumour incidence* in Patulin-treated group
Mean survival time (days)	775 ± 123	771 ± 181
All tumours (total)	42	37
Malignant (total)	13	12
Benign (total)	29	25
Malignant tumours		
Bronchial	0	1
Mammary gland adenocarcinoma	7	8
Adenocarcinoma with lung metastases	0	1
Liver histocytoma with lung metastases	1	0
Reticulum-cell sarcoma of liver, spleen and heart	0	1
Adenocarcinoma of peritoneal cavity	1	0
Arrhenoblastoma	1	1
Renal adenocarcinoma	1	0
Carcinoma of adrenal cortex	1	0
Poorly differentiated sarcoma	1	0
Benign tumours		
Hypophyseal adenoma	36	27
Mammary gland adenofibroma	28	22
Forestomach papilloma	0	4
Glandular stomach adenoma	0	2
Ovarian cystadenofibroma	3	2
Thymoma	0	1
Phaeochromocytoma	0	2
Adenoma of adrenal cortex	5	1
Uterine leiomyoma	1	0
Uterine leiomyoma adenoma	0	1

*No. of rats affected per group of 50.

Table 2. *Survival and tumour incidence in mice treated with 4 mg patulin/kg/day on days 14–19 of gestation*

Type of tumour	Mean survival time (days)....	Tumour incidence* in Control group 575 ± 186	Tumour incidence* in Patulin-treated group 386 ± 161
All tumours (total)		2	5
Specific tumours			
Lymphocytic leukaemia		0	3
Haemangioendothelioma with lung metastases		0	1
Reticulum-cell sarcoma		0	1
Haemangioma of liver		1	0
Haemangioma of spleen		1	0

*No. of mice affected per group of 12.

slight difference in the numbers of rats with hypophyseal adenoma in the control (36) and patulin-treated groups (27) was not significant by the chi-square test at α 0·05. The adenomas of the hypophysis were predominantly basophilic adenomas (25 in the control group and 19 in the patulin-treated group); chromophobic adenomas (seven and six, respectively) and mixed-cell adenomas (four and two, respectively) were rare. The other types of neoplasm recorded are listed in Table 1. Their frequently and latency showed no significant differences between the control and patulin-treated rats, although several in the test group (notably the four forestomach papillomas and two adenomas of the glandular stomach) involved sites and tissues not commonly represented among the lesions observed in control rats of this strain.

Mouse study

Tables 2 and 3 compare the results obtained following oral treatment of Swiss mice, with 2 × 2 mg patu-

Table 3. *Survival and tumour incidence in the offspring of mice treated with 4 mg patulin/kg/day on days 14–19 of gestation*

Survival parameter or type of tumour	Survival and tumour data in Control group Males	Control group Females	Patulin-treated group Males	Patulin-treated group Females
Total of offspring born	40	54	43	52
No. of deaths by day 6 after birth	0	0	8	11
Mean survival time (days)	561 ± 201	617 ± 173	595 ± 197	625 ± 147
No. of tumour-bearing mice	24 (60%)	31 (58%)	15 (43%)	21 (51%)
No. of mice with malignant tumours	22 (55%)	23 (43%)	12 (34%)	18 (44%)
No. of mice with benign tumours	2 (5%)	8 (15%)	3 (9%)	3 (7%)
Specific types of tumour (no. of mice affected)				
Lymphocytic leukaemia	9	9	7	3
Myelocytic leukaemia	3	2	2	4
Plasmocytoma	0	1	0	0
Reticulum-cell sarcoma	1	0	1	0
Osteogenic sarcoma + LM	0	0	0	1
Mammary adenocarcinoma	0	4	0	0
Mammary fibrosarcoma + LM	0	0	0	1
Histiocytoma	0	1	0	0
Pulmonary adenocarcinoma	2	5	0	4
Colonic adenocarcinoma	1	0	0	0
Ovarian cystic adenocarcinoma	0	1	0	0
Arrhenoblastoma	0	1	0	0
Hepatocellular carcinoma	0	1	0	0
Hepatocellular carcinoma + LM	0	0	1	0
Haemangioendothelioma	2	0	0	2
Squamous carcinoma of skin	0	1	0	2
Fibrosarcoma	5	0	1	1
Pulmonary adenoma	2	8	2	2
Cholangioma	0	1	0	0
Haemangioma	0	1	0	0
Uterine leiomyoma	0	1	0	0
Mammary fibroadenoma	0	1	0	0
Forestomach papilloma	0	0	1	0
Skin papilloma	0	0	0	1

+ LM = With lung metastases.

lin/kg daily on days 14–20 of pregnancy, with those recorded for the control group and their offspring. The average survival time of the control group differed markedly from that of the patulin-treated group (575 ± 186 *v.* 386 ± 161 days), although one patulin-treated dam that died of pulmonary oedema 12 days after the birth of its young was excluded from the calculation. In spite of the low mean survival time of the patulin-treated group, five of the eleven dams developed malignant tumours, whereas in the control group only two of the twelve dams bore tumours and these were benign. The malignant tumours were predominatly of lymphoreticular origin.

The group of offspring exposed to patulin transplacentally was reduced by the death of eleven female and eight male suckling mice within 2–6 days of parturition. Gross examination of these dead mice showed haemorrhage in the skin (predominantly in the skin of the head) and to some extent also in the brain and the lung. Histological examination revealed massive hyperaemia in these organs without capillary bleeding. In contrast, none of the offspring in the control group died during the suckling period. The mice that died during this period were excluded from the calculation of the average survival times for male and female offspring. These survival times did not differ significantly between the control mice and those treated with patulin *in utero*. The smaller number of tumour-bearing mice in the latter group compared with the corresponding controls (Table 2) was mainly a reflection of the smaller number of surviving animals, as can be seen from the percentage incidence indicated in parenthesis. While there were no significant differences in the proportions of tumour-bearing mice in the control and transplacentally treated groups, it was evident that the mice in the latter group developed only one type of tumour, whereas in those of the control group two or three different types of tumour sometimes occurred.

DISCUSSION

The strain of rat used in this study shows a high incidence of tumours, particularly of certain benign forms. It was considered suitable, therefore, for use in testing patulin for possibly weak carcinogenic activity. However, the dose level administered, which provided a total oral intake of 358 mg/kg over the 64 wk of the study had no demonstrable carcinogenic effect in the Sprague–Dawley rats used. Four papillomas in the forestomach and two adenomas of the glandular stomach occurred in the patulin-treated group without any similar tumours in the controls but, on the other hand, 36 adenomas of the hypophysis developed in the rats of the control group compared with 27 in the patulin-treated group. Too much emphasis should not be placed on the occurrence of six lesions of the epithelium at the site of application of the test substance; the number of these lesions seems too small and the overall incidence of benign tumours too high for any connection with the patulin treatment to be assumed. The amount of patulin isolated for this study was insufficient for the treatment of a second group of rats with a doubled dosage of patulin.

The shortage of test material was also the reason for carrying out the transplacental study in mice instead of rats. This study demonstrated several malignant lymphoreticular tumours in the treated dams in contrast to the benign lesions seen in the control group. However, it is no longer considered reasonable for changes in small groups of animals to be taken as proof of the carcinogenic action of a substance. A varying incidence of malignancies in such small groups of animals must be regarded as an insignificant event, even though the reduction in the lifespan of these patulin-treated mice was in line with observations of a reduced survival time following treatment with alkylating agents (Schmähl, 1967; Schmähl & Osswald, 1970). Patulin can be regarded as an alkylating agent (Druckrey, Kruse, Preussmann, Ivankovic & Landschütz, 1970).

The slightly diminished number of tumours in the offspring of the patulin-treated mice corroborates the assumption that the dosage used in this study was not carcinogenic. The possible objection that the substance may not have crossed the placenta to penetrate into the embryos does not correlate with the deaths that occurred in this group of offspring between day 2 and 6 after parturition. In spite of this patulin-related toxic effect in the offspring, a decrease rather than an increase in malignant lesions occurred during the lifespan of the survivors of this group.

The studies do not permit any evaluation of a threshold dose of patulin. Several factors (the varying stability of patulin at the different pH values encountered in the gastro-intestinal tract, the inactivation of patulin in the stomach by foodstuffs containing thiol groups, the mucous barrier of the stomach, and patulin inactivation by thiol-containing amino acids or peptides on the surface and within the epithelium of the gastro-intestinal tract) may influence the activity of patulin. On the other hand, achylia must be considered as a probably favourable condition for the expression of a local carcinogenic effect from ingested patulin.

Our negative findings with regard to the carcinogenicity of patulin administered orally to rats and mice should not encourage assumptions about the safety of other mycotoxins present in foods. While the presence of trace amounts of some mycotoxins in certain foods may be unavoidable—a fact recognized by the maximum allowable concentration established in many countries for aflatoxin in laboratory diets, animal feeds and foods for human consumption—the potential danger of producing pulp or juices from more or less rotten fruits (often designated 'slightly infected') must be recognized, so that legislative authorities will take steps to control this practice in countries where it is operated commercially.

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MULTIGENERATION FEEDING STUDIES IN MICE FOR SAFETY EVALUATION OF THE MICROALGA, *SCENEDESMUS ACUTUS*. I. BIOLOGICAL AND HAEMATOLOGICAL DATA

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Abstract—Seven generations of mice of both sexes were given a diet containing 20% drum-dried alga, for 80 wk. The alga (*Scenedesmus acutus*, strain 276-3a) containing 50% protein, was substituted for some of the protein-rich constituents of the basic diet. The body weight, feed efficiency, reproductive capacity, life-span, organ weights, haematology and blood chemistry of the test animals were compared with those of controls. Body weights were increased by the algal diet by 10% over the controls, litter size was decreased by 11%, mean birth weights of pups increased by 4% and survival of female mice was increased by 48%. In older females, the liver-to-body weight ratio increased by 15%, absolute spleen weight increased by 19% and absolute kidney weight increased by 13%. There were no other significant differences between the test animals and the controls. Some of these differences may have been caused by the altered feed composition rather than the algal supplement specifically. The increased feed intake of the test group may also have contributed to the differences.

INTRODUCTION

Ever since the early study by Jorgensen & Convit (1953) there have been suggestions that protein-rich microalgae could be used either as a direct protein source for man or indirectly by feeding them to animals. It was found that when properly processed, some species of the green algae genera Chlorella and Coelastrum, and particularly of the genus Scenedesmus, had a high protein digestibility in several animal species (Becker, Venkataraman & Khanum, 1976; Meffert & Pabst, 1963; Mitsuda, 1965; Pabst, 1974 & 1975; Uesaka, 1965). The unmixed crude protein of these microalgae was found to be of good quality, superior to most other plant proteins (Bock & Wünsche, 1967; Fink & Herold, 1957; Kraut, Jekat & Pabst, 1966; Walz, Koch & Brune, 1975; Witt & Schröder, 1967). Such favourable findings were corroborated by similarly encouraging results from algal feeding tests in volunteers (Kofranyi & Jekat, 1967; Müller-Wecker & Kofranyi, 1973).

Although some of the studies quoted above were extended over periods of several months, their main purpose was the evaluation of the nutritional quality of microalgae as a potential food and/or feed component. In this paper the emphasis is, for the first time, on the toxicological aspects of the intake of the green microalga *Scenedesmus acutus*.

Groups of female and male mice each received, for 80 wk, either a control diet or a diet in which 20% of the basic constituents (mainly protein carriers) were replaced by drum-dried microalgae (*S. acutus*) containing about 50% crude protein. This feeding schedule was used on seven animal generations, the entire experiment lasting for about 3 yr.

EXPERIMENTAL

Preparation of algae. Scenedesmus acutus (strain 276-3a of the culture collection at the Pflanzenphysiologisches Institut der Universität Göttingen) was produced autotrophically as described by Stengel (1970) and Soeder (1976) in the experimental field plant at Dortmund. The alga was cultivated for extended periods, out of doors, in shallow ponds of 80–200 m^2 net surface (water depth 15–20 cm). The nutrients supplied consisted of a mineral mix, urea and gaseous CO_2. After harvesting by centrifugation, the resulting algal slurry was processed to a dry powder by heating on a drum drier at 110–130°C for about 10 sec. This treatment makes the algal material fully digestible and sterilizes it to an appreciable extent so that it can be stored for a sufficiently long period. The algal powder had a residual moisture content of 4–8% and an average crude protein (N × 6·25) content of 50–56% (the complete analysis is given by Soeder & Pabst, 1970). In addition to the essential fertilizer ingredients, the dry algal product contained environmental contaminants derived from the tap-water, from the fertilizer and from air pollutants (Payer, Runkel, Schramel, Stengel, Bhumiratana & Soeder, 1976; Payer, Soeder, Kunte, Karuwanna, Nonhof & Graf, 1975). During the overall experimental period of 3 yr, nine different batches of *S. acutus* powder of essentially similar chemical composition were produced and used for the preparation of pelleted diets.

Diets. The control groups were fed a pelleted commercial stock diet for mice ("ssniff M", Versuchstier-Diäten GmbH, Soest). Its composition and that of the test diet are summarized in Table 1. The algal diet contained 20% *S. acutus* powder replacing the

Table 1. *Comparative composition of diets (% of air dry weight)*

Components	Control diet	Algal-supplemented diet
Scenedesmus powder (drum dried)	—	20·0
Soya-bean meal (extracted, toasted)	10·0	—
Fish meal	8·0	5·0
Fish solubles	2·0	2·0
Rolled oats	30·0	30·0
Ground cereals (wheat, maize, barley, oats)	24·0	20·0
Rusk meal	5·0	5·0
Tapioca meal	5·0	5·0
Wheat bran	4·0	4·0
Wheat germ	3·0	3·0
Alfalfa meal	3·0	—
Distillers solubles	2·5	2·5
Whey powder	2·2	2·2
Vitamin mixture	0·2	0·2
Mineral mixture	1·1	1·1

extracted and toasted soya-bean meal, the alfalfa meal, part of the fish meal and part of the ground cereals. Both diets were manufactured by the same company and, except for those fed to the first two generations, both types of pellets were identical in size and hardness. The earlier batches of algal pellets were too hard.

Because of the algal concentration in the experimental diet, the crude protein content (25·0 ± 1·8%) of the dry pelleted material was somewhat higher than that of the control diet (22·4 ± 1·2%). However both feeds were approximately isocaloric: 325 kcal/100 g, using the Rubner factors (Bender, 1968) for calculation. The residual water content of both diets averaged around 10%. Both diet and tapwater were given *ad lib.*

Animals. The limited space available and shortage of alga led us to use mice rather than rats. The starter animals of the mouse strain NMRI/HAN were obtained from the Zentralinstitut für Versuchstiere (Hannover). The mice were conventional (non-SPF) and were maintained in groups of five per Macrolon® cage (standard type 3), each group remaining together throughout life except at mating times.

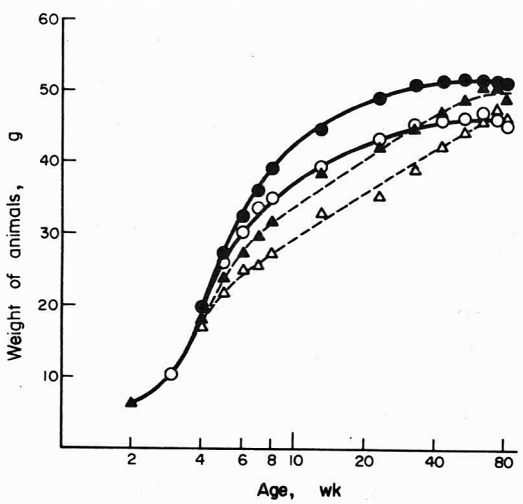

Fig. 1. Mean body weights of generations F_2–F_6 of male (●, ○) and female (▲, △) mice fed algal (●, ▲) or control (○, △) diet.

Once a week the animals were weighed individually, and the cages were disinfected with Lysolin® (Schülke & Mayr GmbH, Hamburg/Norderstedt) and provided with fresh litter consisting of the wood-meal ssniff® bedding (Versuchstier-Diäten GmbH) and the adsorbent Florideal® (Chemie-Mineralien GmbH, Bremen). The animal room was kept at 23 ± 2°C and at a relative humidity above 50%. The experiment was carried out over seven generations (F_0–F_6) without interruption. Except for the F_0 and F_1 generations, which comprised smaller numbers of animals, the two groups of every generation each consisted of 20 males and 20 females. At the age of 11–12 wk (in the case of generations F_3 and F_5 as early as 7 wk), the animals were mated. Those of generations F_5 and F_6 were mated for a second time at the age of 30 wk, in order to test the fertility of the older animals.

The pups were weaned at 3 wk. From the offspring of each of the two groups, those of similar weight and birth date were selected for the following generation. Most animals were killed at about 80 wk. In some generations an additional experimental group was kept from the initial surplus of the population and killed earlier than the others; of these, group 1, comprising 20 males and 20 females from the F_4 generation, was killed after 10 wk, group 2, comprising 15 males and 20 females from the F_3 generation, was killed after 40 wk, and group 3, comprising 13 males and 20 females of the F_5 and F_6 generations, was killed after 52 wk.

Investigations. Animals were killed under ether anaesthesia, as much blood as possible being removed by open chest heart puncture using heparin as an anticoagulant. Haematological and blood-chemical values (haemoglobin, haematocrit, blood glucose, urea, protein and transaminases) were determined by standard clinical methods. The liver, spleen, kidneys, brain and testes were macroscopically inspected and weighed. Thereafter, these and all the other major organs and tissues were fixed in neutral formaldehyde solution for the histo-pathological examination, which was carried out at the Institut für Pathologie der Tierärztlichen Hochschule, Hannover.

Statistical evaluations. With the exception of survival rates, the means and standard errors were calculated for all data. The statistical significance of

Table 2. *Reproduction data for mice of seven generations given diet containing 20% alga or control diet*

Generation	Age at mating (wk)	No. of females			Mean litter size			Mean body weight of pups at	
		Mated	Pregnant	With live litters	At birth	on day 4	on day 14	Birth	Day 14
				Controls					
F_0	12	18	18	17	10·06	8·06	7·12	1·46	8·41
F_1	12	10	10	10	10·20	9·60	9·20	1·46	8·07
F_2	11	20	18	18	10·06	7·39	7·39	1·49	8·85
F_3	7	20	20	20	9·95	8·70	8·70	1·46	7·95
F_4	12	20	20	20	10·20	7·60	7·60	1·44	7·94
F_5	7	20	19	19	10·00	8·84	8·68	1·47	7·83
F_6	11	20	20	20	9·45	8·80	8·60	1·49	7·88
F_5*	30	20	20	17	8·23	7·59	7·58	1·45	7·85
F_6*	30	19	19	16	8·00	7·63	7·63	1·46	8·56
				Animals on algal diet					
F_0	12	18	17	17	7·88	(4·24)	(3·82)	1·53	8·42
F_1	12	10	10	10	8·60	8·10	7·80	1·54	8·40
F_2	11	20	18	18	9.72	8·11	8·11	1·52	8·46
F_3	7	20	20	19	9·42	7·68	7·68	1·55	8·51
F_4	12	20	19	19	9·42	8·42	8·42	1·51	8·32
F_5	7	20	(15)	(14)	9·79	7·93	7·50	1·51	7·96
F_6	11	20	19	19	7·74	6·37	6·21	1·56	8·81
F_5*	30	20	20	16	7·63	6·69	6·69	1·61	8·28
F_6*	30	20	18	18	7·22	6·50	6·50	1·67	10·14

Values in parentheses were considered anomalous and not included in the calculations of reproductive indexes (Table 5).
*Second mating.

differences between animals of the same generation and sex was calculated by Student's t test (Sachs, 1968). Outsider values were eliminated by the test of Nalimov and Grubbs (Kaiser & Gottschalk, 1975). In some cases, data within comparable groups were paired in order to calculate the significance of differences between pairs by the t test (Kaiser & Gottschalk, 1975).

RESULTS

Growth and food consumption

The mean body weights at a given age for generations F_2–F_6 are plotted in Fig. 1. In these generations there was a statistically significant increase, of about 10% in growth rate in animals of both sexes fed the algal diet compared with the corresponding control groups. This effect was not apparent in the first two generations, in which the body weights of both males and females on the algal diet were only slightly greater or in some cases lower than those of the controls. This effect was attributed to a reduced feed intake due to excessive hardness of the test-diet pellets and the F_0 and F_1 generations were therefore excluded from the growth-rate evaluation. Food consumption was higher in the algal-fed mice of the F_2–F_6 generations than in controls. In other experiments involving a restricted feeding schedule there was no difference in weight gain and development between the test and control groups.

There were no consistent or statistically significant differences between groups or generations in the feed

Table 3. *Mean indexes of reproduction (first matings only) for F_0–F_6 generations of rats*

Parameter	Controls	Alg-group
No. of newborn/litter	9·99	8·94**
Birthweight/pup (g)	1·47	1·53***
Index of fertility† (%)	97·4	95·4
Index of gestation‡ (%)	99·2	99·0
Index of viability§ (%)	83·8	84·7
Index of lactation‖ (%)	97·4	98·4
Total litter weight at birth/dam (g)	14·61	13·68
Weight-gain of litter at weaning (g)	54·55	52·33

†Index of fertility = The proportion of matings resulting in pregnancy.
‡Index of gestation = The proportion of pregnancies resulting in live litters.
§Index of viability = The proportion of pups born which are alive at 4 days.
‖Index of lactation = The proportion of pups alive at 4 days which survive to weaning.
Values marked with asterisks differ significantly from the corresponding controls: **$P < 0·01$; ***$P < 0·001$.

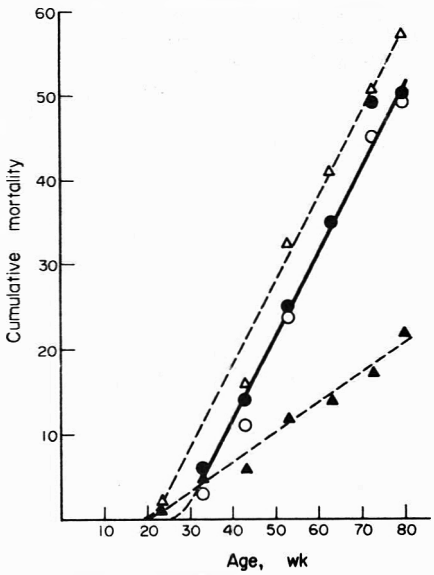

Fig. 2. Cumulative death rates of male (●, ○) and female (▲, △) mice fed algal (●, ▲) or control (○, △) diet.

efficiency ratio (increase in body weight (g)/total feed intake (g) determined over wk 4–7 for each generation).

Reproduction

The data from which the reproduction indexes (Protein-Calorie Advisory Group, 1970) were calculated are presented in Table 2. In generations F_5 and F_6 the animals were mated for a second time at the age of 30 wk. Although they followed the general trends, second litters of F_5 and F_6 were not included in the calculation of the reproduction indexes (Table 3). The dams on the algal diet produced significantly smaller litters (−11%) than the animals of the control groups. However there was an increase in mean birth weight (+4%) in the algal-fed groups. The gestation, viability and lactation indexes were similar in both the algal and the control groups (Table 3).

Survival

The cumulative numbers of animals that died before wk 80 are summarized in Fig. 2. Throughout the experiment the average duration of life for males was the same in the test and control groups. By contrast, the algal-fed females displayed a highly significant increase in life expectancy. After 80 wk the number of surviving females was nearly 50% higher than in the other groups (control females and males of both groups), in which average mortality was considered to be normal for conventional mice (Zentralinstitut für Versuchstiere, 1977).

Organ weights

The weights of liver, spleen, kidneys, brain and testes are summarized in Tables 4 and 5. In general the organs were taken from 80-wk-old animals and the mean weights were determined over the seven generations. In some generations organ weights were determined in younger animals from the parallel groups. In the latter, no significant deviations were detected for brain, spleen, kidney or testes, but the liver weights showed highly significant differences between the two groups. The high liver weights of the groups on the algal diet, especially in young animals, remained higher than those of the controls when expressed as a percentage of body weight but the differences were less marked; the increases over controls were 20% (female) and 11% (male) for 10-wk-old mice and 15% for female but none for male 80-wk-old mice. Higher weights of spleen (19% increase) and kidneys (13% increase) in the groups receiving algae were restricted to older females.

Haematological data

The results of the haematological investigations are presented in Table 6 as mean values for the seven experimental generations. The only detectable difference between the test and control groups was a 3% reduction of haemoglobin and haematocrit in the algal-fed female mice.

DISCUSSION

Several multigeneration experiments have already been carried out for the toxicological evaluation of non-autotrophic single-cell proteins, mainly in rats, but also in the target (farm) animals. Both with yeasts grown in different media (de Groot, 1976; Shacklady, 1975) and with bacteria grown in methanol (Stringer

Table 4. *Absolute and relative liver weights of mice fed an algal-diet or control diet*

Age (wk)	Generation	Males		Females	
		Controls	Animals on algal diet	Controls	Animals on algal diet
		Liver weight (g)			
10	F_4	1·94 ± 0·25 (20)	2·39*** ± 0·26 (20)	1·31 ± 0·19 (20)	1·93*** ± 0·26 (20)
40	F_3	2·79 ± 0·23 (15)	3·50*** ± 0·50 (9)	1·64 ± 0·36 (15)	2·29*** ± 0·26 (13)
52	F_5 & F_6	2·36 ± 0·35 (12)	2·95 ± 0·93 (8)	2·36 ± 0·35 (19)	2·32* ± 0·24 (16)
80	F_0–F_6	2·86 ± 0·80 (67)	3·0 ± 0·64 (59)	2·11 ± 0·43 (69)	2·50*** ± 0·54 (104)
		Relative liver weight (% of body weight)			
10	F_4	5·07 ± 0·57 (20)	5·64** ± 0·48 (20)	4·41 ± 0·34 (20)	5·31*** ± 0·64 (20)
40	F_3	6·30 ± 0·76 (15)	6·90 ± 0·58 (9)	5·03 ± 0·64 (15)	5·11 ± 0·37 (13)
52	F_5 & F_6	5·99 ± 0·61 (12)	6·74* ± 0·91 (8)	5·01 ± 1·24 (19)	4·84 ± 0·46 (16)
80	F_0–F_6	6·41 ± 1·21 (67)	6·35 ± 1·15 (59)	4·72 ± 0·78 (69)	5·42*** ± 0·90 (104)

Values are means ± SEM with the number of animals used in parentheses. Values marked with asterisks differ significantly (Student's t test) from the corresponding controls: * $P < 0·05$; ** $P < 0·01$; *** $P < 0·001$.

Table 5. *Mean weights of brain, kidneys, spleen and testes of 80-wk-old mice (F_0–F_6) fed on algal or control diet*

Organ	Males		Females	
	Controls	Animals on algal diet	Controls	Animals on algal diet
Brain	0·52 ± 0·02 (74)	0·54 ± 0·02 (73)	0·53 ± 0·03 (69)	0·54 ± 0·02 (106)
Kidneys	0·71 ± 0·11 (73)	0·75 ± 0·12 (72)	0·44 ± 0·06 (69)	0·50* ± 0·08 (104)
Spleen	0·15 ± 0·05 (66)	0·14 ± 0·04 (66)	0·15 ± 0·05 (58)	0·18* ± 0·05 (98)
Testes	0·24 ± 0·03 (73)	0·22 ± 0·03 (73)	—	—

Values are means ± SEM with the numbers of animals in parentheses. Values marked with an asterisk differ significantly (Student's t test) from the corresponding controls: $*P < 0.001$.

Table 6. *Blood determinations for 80-wk-old mice of all seven generations fed algal or control diet*

Parameter	Males		Females	
	Controls	Animals on algal diet	Controls	Animals on algal diet
Haemoglobin (g/100 ml)	12·8 ± 1·6 (72)	12·7 ± 1·4 (69)	13·8 ± 1·2 (68)	13·2 ± 1·3 (104)
Haematocrit (% v)	39·5 ± 4·3 (72)	39·0 ± 3·4 (69)	41·9 ± 3·2 (68)	40·5 ± 3·1 (104)
Leucocyte count (10^3/mm^3)	4·45 ± 1·67 (70)	4·48 ± 1·88 (70)	3·14 ± 1·41 (68)	3·29 ± 1·41 (100)
Glucose (mg/100 ml plasma)	159 ± 31 (72)	172 ± 32 (73)	177 ± 22 (60)	186 ± 23 (96)
Urea (mg/100 ml plasma)	49 ± 12 (61)	49 ± 10 (66)	47 ± 11 (59)	46 ± 9 (96)
Plasma protein (g/100 ml)	5·67 ± 0·67 (72)	5·91 ± 0·63 (73)	5·60 ± 0·63 (59)	5·49 ± 0·51 (96)
Plasma GOT (mU/ml)	33 ± 12 (63)	33 ± 12 (65)	37 ± 11 (51)	37 ± 12 (87)
Plasma GPT (mU/ml)	29 ± 18 (54)	30 ± 17 (67)	21 ± 12 (51)	22 ± 12 (89)

GOT = Glutamic-oxalic transaminase GPT = Glutamic-pyruvic transaminase.
Values are means ± SEM with the numbers of animals in parentheses.

& Wilson, 1976) no adverse effects were found. Other authors (Ågren, Stenram, Nordgren, Eklung & Glas, 1974) found a lower growth rate and increased occurrence of renal calcification in rats fed yeasts, and even more severe effects, such as weight loss and increased mortality, in rats fed bacterial protein, grown on a chemically pure hydrocarbon fraction, as the sole source of dietary protein. Dietary imbalances may have been responsible for these unfavourable results (de Groot, 1976).

To our knowledge, long-term testing of microalgae has so far been performed only with blue-green algae (Cyanophyceae) of the genus Spirulina (Boudene, Collas & Jenkins, 1975). The test was carried out on rats and lasted for 75 wk. However only 25% of the total feed *protein* consisted of dried, atomized algae and the test was not continued over several generations. Except for a slight decrease in the weight of the females for a time, no differences were observed, in comparison with the control animals fed a casein diet.

Our multigeneration test in mice was intended as a further step towards evaluating the toxicological safety of the ubiquitous green alga *S. acutus*. For this group of algae (Chlorophyceae) toxicity or toxin production—such as that occurring in a few microalgae of other groups (Hellebust, 1974; Schantz, 1973)—has never been reported. The encouraging results of numerous feeding trials with *S. acutus* (strain 276-3a) cited in the introduction, lend support to this statement.

The data presented in this paper are not by themselves conclusive. The results of the histopathological investigations will be dealt with in a subsequent publication. It should also be emphasized, that the Scenedesmus biomass produced in open-air cultures in the industrial city of Dortmund contains higher concentrations of toxic minerals (Payer *et al.* 1976) and polycyclic hydrocarbons (Payer *et al.* 1975) than is typical for algae and commercial feedstuffs cultivated in less industrialized regions. Furthermore the dietary concentration of the microalga used in this study was substantially higher than would be expected if *S. acutus* were used to feed farm animals.

In evaluating the differences between the mice given algal diet and the controls, one must keep in mind that commercial feedstuffs, such as that fed to the control animals, must be considered the best for breeding, growth and maintenance as a result of experience and testing over many years. The insertion of as much as 20% algal powder in such a balanced diet involves a considerable alteration in the individual nutrients and in the crude-fibre content. This alone could be responsible for the alterations in weight of those organs that are characterized by intensive metabolism. The increased body weight of the mice fed algal diet was attributable to a higher feed intake, and the reduced litter size may have been caused by the higher weight of the dams; it is well known that fat females give birth to smaller litters, but heavier young. No explanation can be offered for the prolonged lifespan of the algal-fed females. In an incomplete multigeneration experiment with rats, which has so far included five generations, none of the findings described here for mice have been observed. We therefore consider the mouse a more sensitive test organism than the rat for this purpose.

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Review Section

ESTIMATION OF TOXIC HAZARD—A DECISION TREE APPROACH

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Summary—Safety evaluation is caught in a frustrating circle. It is neither possible nor sensible to try to obtain the information needed to assess every imaginable toxic risk associated with every substance, and pursuit of greater safety therefore demands the setting of priorities as well as sensible limits for investigation. To do this with confidence requires possessing the very information that is lacking and that can be won only slowly on a few substances at a time, with significant uncertainty and at considerable cost. This requires priorities, and completes the circle of frustration. Individual toxicologists deal with this problem by using 'experience', a personal synthesis of accumulated knowledge of structure-activity relationships, metabolic mechanisms, chemical reactivity, human exposure and other relevant information. Such expert judgement is often very effective in distinguishing potential risks worth pursuing from problems on which effort would be wasted but, because it is usually so inexplicit and subjective, it is seldom able to invoke the public confidence most decisions now require.

This paper proposes a procedure for making a significant part of this process rational, public and explicit. It uses much currently available toxicological data to validate the procedure, which consists of a 'decision tree' of 33 questions, each answered 'yes' or 'no'. Each answer leads to another question or to final classification into one of three classes (I, II and III) reflecting a presumption of low, moderate or serious toxicity. The tree is organized into branches dealing with major chemical classifications and is intended for use with all ingested, structurally defined organic and metallo-organic substances. Answering the questions requires chemical or biochemical training, and relies primarily on features of chemical structure. Occurrence in body tissues and fluids, and natural occurrence in food are also involved. The logic of the tree rests heavily on known data on metabolism and toxicity. The classification according to presumptive toxicity can be combined with knowledge of human intake to provide for each substance a 'protection index', which can be used to establish priorities and to define tentatively the extent of appropriate testing. The procedure has been applied to a large number of pesticides, drugs, food additives and industrial and environmental chemicals of known biological properties. So far it has not resulted in any underestimation of toxicity, and it appears to provide a practical means for discriminating effectively among different levels of probable hazard.

Background

Never since toxicology first emerged as a distinct field of applied science have the resources within the field been adequate to meet the increasing demands made on them. In part, this has been due simply to the advance of all science (Price & Desolla, 1969). W. G. Galetto (personal communication 1977) has pointed out that a count of the number of both chemical abstracts and biological abstracts between 1906 and 1976 shows an exponential growth with a doubling period of about 12 years. *Biological Abstracts* published 1,498,000 items in 1965 and about 3,000,000 in 1977. Once well-established, conclusions seldom remain undisturbed for long.

Toxicology, however, bears a double burden. We demand ever greater safety in an environment that many people fear—not necessarily correctly—is increasingly hazardous. At the same time, the notably rapid advance of analytical chemistry has continued to alert us to the presence of previously unsuspected toxicants and to a host of substances we have not yet begun to evaluate. It is clear even to a casual observer that the sensitivity and precision of analytical chemistry are far ahead of toxicology. Even less happily, toxicologists find it far easier to produce an adverse effect in laboratory animals than to interpret with assurance the meaning of such an effect for human safety.

Our response to this situation has customarily been simply to demand more testing on virtually every substance that has recently been evaluated. No one denies that we would be better off with more data. Our universal problem has been that the demand for data has grown faster than the supply. The recent passage of the Toxic Substances Control Act merely carries this trend further. At least several million dollars, in each case, have been spent on toxicological work on cyclamates, on saccharin, on Red No. 2 and on monosodium glutamate. Yet it is clear, at least in the minds of some, that from a regulatory standpoint the safety of these long-used ingredients is far from well established. Obviously, we cannot devote to every substance of present or potential interest even a fraction of this kind of effort.

This suggests some rational establishment of priorities, as a guide to the sequence, scope and intensity of investigative effort. This has several times been urged, but usually in the general terms of looking first at those substances whose structure or known biological effects raise questions, and then at the substances used in largest quantity (Joint FAO/WHO Expert Committee on Food Additives, 1976; Panel on Chemicals and Health, 1973; Select Committee on Flavor Evaluation Criteria. 1976). This is rational as far as it goes, but it is an extremely coarse screen, which fails to differentiate at all among less serious sources of potential hazard. By missing this opportunity, it leads either to the disregard of hazards of low or intermediate degree or to the unnecessary expenditure of effort on the trivial as well as the more significant.

We require therefore a preliminary assessment of probable risk—not as a substitute for data but as a guide to the priority and scope of the effort required to acquire more information. This paper attempts to suggest how information on intake currently or potentially available can be combined with a preliminary assessment of probable toxicity to provide such a guide.

We are aware of a recent publication proposing a system for selecting and defining priorities for chemicals that may present environmental hazards (Arthur D. Little, Inc., 1977). While that publication differs markedly from our paper in its scope, objectives and content, it approaches many of the same considerations with parallel concepts and language.

Toxicity has been defined as "the capacity of a substance to produce injury" and hazard as "the probability that injury will result from the use of a substance in a proposed quantity and manner" (Food Protection Committee, 1970). The primary consideration is quantity—for ingested substances, intake. Safety (the inverse of hazard) "is the practical certainty that injury will not result from the substance when used in the quantity and in the manner proposed for its use" (Food Protection Committee, 1970).

Assessment of risk, therefore, has two principal components: exposure and toxicity. There are other important considerations, including variation in individual susceptibility and the possibilities of synergistic or antagonistic effects, but in a preliminary assessment of the safety of ingested materials, intake and toxicity are paramount.

Estimates of intake of the chemical substances found naturally or introduced intentionally into food are available or potentially available largely because of the work of several agencies and organizations combined in the reports of the Subcommittee on Review of the GRAS List—Phase II (1972) of the National Academy of Sciences. Estimates of indirect-additive intakes are more difficult. The market-basket survey of the Food and Drug Administration provides actual data on pesticide residues (Food and Drug Administration Bureau of Foods, 1977). Packaging component intakes can sometimes be estimated from extraction studies that simulate 'worst cases'. Intakes from over-the-counter drugs are less readily available but those from prescription drugs are more closely controlled, although not necessarily known.

Toxicologists and pharmacologists customarily make preliminary judgements of probable biological effects, most commonly in deciding what substances to test and what effects to anticipate. Such judgements are based on knowledge or assumptions concerning structure–activity relationships and metabolic fate, as well as on presumptions that may be drawn from the occurrence of a substance in food or in body tissues and fluids. While recognizing that generalizations are difficult, and at times incorrect, we cannot avoid making them if we are to choose sensibly what to work on first and how far to go. What has been lacking is a systematic coding of these distinctions, or at least of those so commonly used that they might reasonably be expected to enjoy wide applicability and support.

This paper is a proposal in the direction of such a coding. By the use of a 'decision tree', it seeks unambiguously to classify every structurally defined organic or metallo-organic chemical by criteria that are based largely on structure or on widely known facts of biochemistry and physiological chemistry. It excludes polymers because they are not structurally defined in terms of chain length, molecular weight and cross-linking. It is concerned only with oral toxicity. It results in the placing of every such substance into one of three classes representing an estimate of toxic threat.

The tree is organized into branches, each of which deals with a broad class of compounds. Some questions are 'sorting' questions, which determine the branch to be followed. All questions lead either to another question or to a final classification. A number of terminal questions deal with aspects common to several classes of compounds and are therefore found at several branch ends. Readers may find the summary flow diagram (Fig. 1), suggested by Dr. B. L. Oser, helpful in viewing the organization and use of the tree. When one combines such an estimate of toxic threat with an estimate, or observation, of exposure, one may obtain the kind of preliminary assessment of probable risk that we are seeking. In the discussion section, we pursue such an assessment.

Class I substances are those with structures and related data suggesting a low order of oral toxicity. If combined with low human exposure, they should enjoy an extremely low priority for investigation. The criteria for adequate evidence of safety would also be minimal. Greater exposures would require proportionately higher priority for more exhaustive study.

Class III substances are those that permit no strong initial presumptions of safety, or that may even suggest significant toxicity. They thus deserve the highest priority for investigation. Particularly when per capita intake is high or a significant subsection of the population has a high intake, the implied hazard would then require the most extensive evidence for safety-in-use.

Class II substances are simply intermediate. They are less clearly innocuous than those of class I, but do not offer the basis either of the positive indication of toxicity or of the lack of knowledge characteristic of those in class III.

A similar classification, but differently derived, has been used in the safety evaluation of flavourings (Oser & Hall, 1977). While the system outlined here is more broadly based, encompassing most ingested substances, the two classifications are wholly compatible.

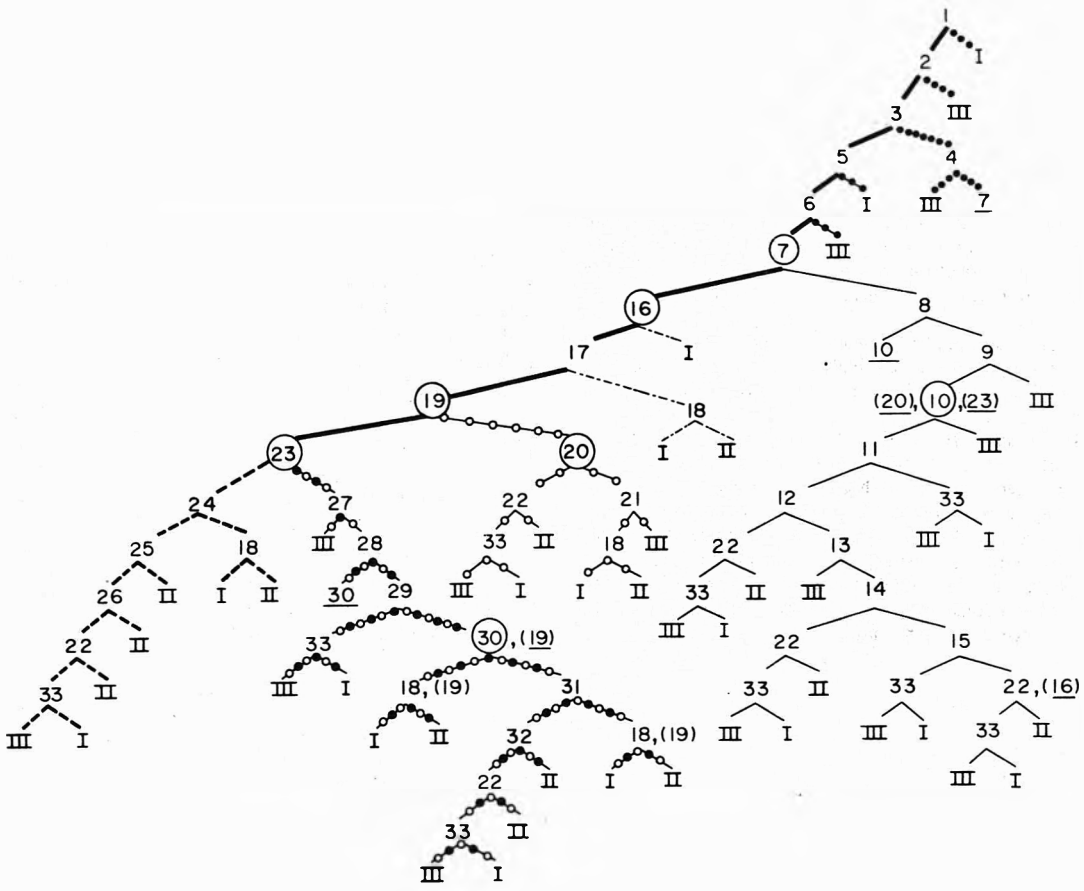


Fig. 1. A schematic diagram of a decision tree for the estimation of probable toxicity. Assessors should (a) start with question 1, (b) proceed by 'no' ↙ or ↘ 'yes', (c) move from any underscored number encountered to same circled number and (d) proceed to final classes I, II or III. Working downwards through the tree, the symbols designate the following groupings: biological normality (●●●), high and low toxicity (●–●–●); heterocyclics (——); terpenoids (–·–·); aliphatics (–O–O–O); aromatics (–O–●–O); alicyclics (– – –).

Definitions for use with the decision tree

The use of this decision tree and these definitions presupposes a working familiarity with organic chemistry, biochemistry and food chemistry. Even individuals with a broad knowledge in these areas will find it useful to consult references such as the *Merck Index* (1976) and the 'Weurman Report' (Central Institute for Nutrition and Food Research TNO, 1973), for detailed information on natural occurrence in food, and *Hawk's Physiological Chemistry* (Oser, 1965).

Users should review these definitions carefully and refer to them frequently. Because they are fashioned to serve the needs of this paper, they differ in several minor, but important, respects from the meanings commonly attributed to these words and phrases. Italicized key words used in the procedure (pp. 258–263) are followed by a letter, in parenthesis, referring to the following definitions:

(A) *Aliphatic* includes olefinic and polyolefinic, but not acetylenic or alicyclic compounds.

(B) *Aromatic* means that the substance has at least one benzene, furan, thiophene, pyridine or pyrrole ring, however substituted and whether or not it is fused to another ring.

(C) *Common component of food.* In something as diverse, changing and occasionally uncertain as natural occurrence, it is only possible to define a guideline, not a firm rule. For this decision tree, the term *common component of food* denotes a substance that has been reported in the recognized literature as occurring in significant quantity (approximately 50 ppm or more) in at least one major food, or in trace quantities at the ppm level or less in several foods, including minor or less frequently consumed foods. The latter include spices, herbs and ethnic specialities. This definition *excludes* natural or man-made contaminants, and hormones.

(D) *Common terpene* means an isoprenoid compound (carbon skeleton made up of two or more 5-carbon isoprene units), reported in the literature as a more than trace constituent of two or more generally consumed foods, either raw or as ordinarily prepared for consumption, without added ingredients.

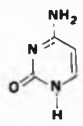
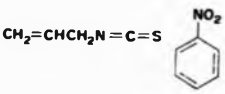
(E) *Functional group* is a portion (sometimes called a radical) of an organic molecule consisting of a combination of atoms of two or more elements (at least one of which is not hydrogen or carbon) and causing the molecule to exhibit a characteristic set of reactions. For the purpose of this classification this definition excludes carbon-carbon double bonds and aromatic rings.

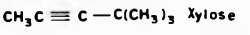
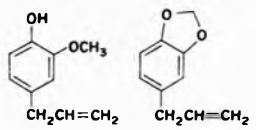
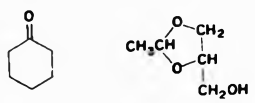
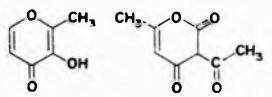
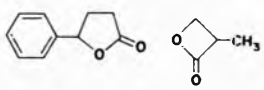
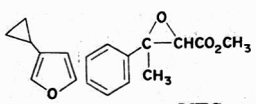
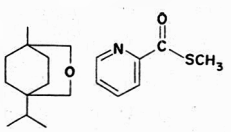
(F) *Normal constituent of the body* means any systemic constituent present at a normal physiological level, whether free or combined, except hormones. This includes essential nutrients and major food constituents and the physiologically normal metabolites of each. It excludes transitory substances present only as a result

of (a) trace constituents of food, (b) gut contents or (c) products of the actions of the gut flora.
(G) *Open chain* means the absence of any ring structure.
(H) *Readily hydrolysed* means known to be or, in the absence of any contra-indication based on structure, assumed to be hydrolysed either during food preparation or by physiological processes after consumption.
(I) *Simply branched* means branched at C–C bonds, with branches of two or more C atoms, at not more than two points along the main chain, with no secondary branching. Multiple branching, consisting only of 1-carbon moieties, falls within this definition of simply branched.
(J) *Sterically hindered* means posing steric hindrance to a functional group equivalent to or greater than that exhibited by *o-tert*-butyl or 2,6-disubstitution on an aromatic ring.
(K) *Structurally closely related* means
(a) a member, not more than two carbon atoms removed, in a homologous series, except ethoxy and higher homologues of a methoxy compound;
(b) a primary alcohol and its aldehyde, or either of these and the corresponding carboxylic acid;
(c) a secondary alcohol and the corresponding ketone;
(d) structural isomers unlikely to involve steric hindrance of a functional group;
(e) compounds with the same functional groups, the remainder of the compound being known to be easily and harmlessly metabolized;
(f) non-sterically hindered esters, thioesters, acetals, ketals and their components.

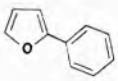
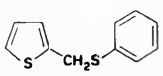
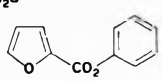
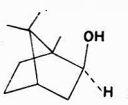
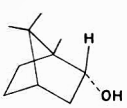
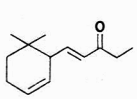
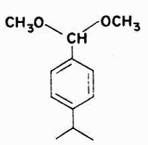
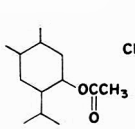
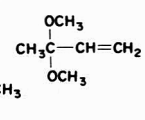
Procedure for the decision tree

In the light of the chemical structure of the substance and using the accompanying definitions, answer the following questions, moving in the order indicated by the question numbers in the 'no' or 'yes' columns until a classification—I, II, or III—is reached. In borderline cases, choose II in preference to I, and III in preference to II. In cases where a structure is subdivided into individual residues, classify the whole structure according to the most conservative classification of the separate residues. In treating functional groups, consider the entire group, not the individual fragments (e.g. a thioamide, $RCSNH_2$, is a *thioamide* not a thione and an amine; a hydrazine, $RNHNH_2$, is not two amines; an anhydride is not an ether and two vicinal ketones).

	If 'no' ....proceed to....	If 'yes'	Examples with answers (and following step or classification)
1. Is the substance a *normal constituent of the body* (F) or an optical isomer of such? This question throws into class I all normal constituents of body tissues and fluids, including normal metabolites. Hormones are excluded, as are, by implication, the metabolites of environmental and food contaminants or those resulting from disease states.	2	I	 NO (2)  YES (Class I)
2. Does the substance contain any of the following functional groups: an *aliphatic* (A) secondary amine or a salt thereof, cyano, *N*-nitroso, diazo (e.g. CH_2N_2), triazeno ($RN{=}NNH_2$) or quaternary nitrogen, except in any of the following forms: $>C{=}N^+R_2$, $>C{=}N^+H_2$ or the hydrochloride or sulphate salt of a primary or tertiary amine? Questions 2, 3 and 4 are a means of placing in class III those structures that contain elements or valency states often associated with enhanced toxicity. Halo-, nitro-, *N*-nitroso- and diazo- compounds, organophosphates, quaternary nitrogen compounds (although note modifying comment), and similar xenobiotic structures should cause 'yes' answers to questions 2 and 3 and a 'no' answer to question 4.	3	III	 NO (3) YES (Class III)
3. Does the structure contain elements other than carbon, hydrogen, oxygen, nitrogen or divalent sulphur?	5	4	 NO (5)  YES (4)
4. Do all elements not listed in question 3 occur only as (a) a sodium, potassium, calcium, magnesium or ammonium salt of a carboxylic acid, or (b) a sulphate or hydrochloride of an amine, or (c) a sodium, potassium or calcium sulphonate, sulphamate or sulphate? (If the answer is yes, treat as the free acid, amine, unsulphonated or unsulphated compound, except for the purposes of questions 24 and 33, and proceed.) This is intended to let through, for further consideration, certain acid, amine, sulphonate and sulphate salts. Sulphamate salts are treated as such because they are not readily hydrolysed.	III	7	$CHCl_3$ NO (Class III)  YES (7)

	If 'no' ....proceed to....	If 'yes'	Examples with answers
5. Is it a *simply branched* (I) acyclic *aliphatic* (A) hydrocarbon or a common carbohydrate? This drops out the generally innocuous hydrocarbons and carbohydrates.	6	I	 Xylose NO (6) YES (Class I)
6. Is the substance a benzene derivative bearing substituents consisting *only* of (a) hydrocarbon chains or 1'-hydroxy or hydroxy ester-substituted hydrocarbon chains and (b) one or more alkoxy groups, one of which must be para to the hydrocarbon chain in (a)? This places in class III safrole. myristicin and related substances.	7	III	 NO (7) YES (Class III)
7. Is the substance heterocyclic?	16	8	 NO (16) YES (8)
8. Is it a lactone or cyclic diester? This question separates the lactones and cyclic diesters from other heterocyclic compounds.	10	9	 NO (10) YES (9)
9. Is it a lactone fused to another ring, or a five- or six-membered α,β-unsaturated lactone? This places certain lactones known or suspected to be of unusual toxicity in class III.	*	III	 NO (23) YES (Class III)
10. Is it a 3-membered heterocycle? This places such substances as the epoxides and ethylenimine in class III.	11	III	 NO (11) YES (Class III)
11. Disregarding only the heteroatoms in any one ring, does that heterocyclic ring contain or bear substituents other than *simply branched* (I) hydrocarbons (including bridged chains and monocyclic aryl or alkyl structures), alkyl alcohols, aldehydes, acetals, ketones, ketals, acids, esters (including cyclic esters other than lactones), mercaptans, sulphides, methyl ethers, hydroxy or single rings (hetero or aryl) with no substituents other than those just listed? Questions 11–15 separate out various categories of heteroaromatic substances. Under 11, set aside and do not consider the atom(s), usually oxygen, nitrogen or sulphur, making the ring heterocyclic. If there is more than one hetero ring, regard each ring separately, with the remainder of the structure as substituents of that hetero ring. Other than the heterocyclic atom(s), does the ring carry anything besides the simple groups listed? If so, the answer is 'yes'	12	33	 NO (12) YES (33)

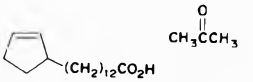
*If it is a lactone, from this point on treat the structure as if it were the hydroxy acid in the form of its more stable tautomer and proceed to question 20 if it is open chain, to 10 if it is heterocyclic and to 23 if it is carbocyclic; if it is a cyclic diester treat as the separate components.

	If 'no' ....proceed to....	If 'yes'	Examples with answers
and the next question 33. If not, then classify further by question 12 *et seq.* Bridged-chain derivatives may be represented by structures like the bicyclic ether 1,4-cineole while monocyclic aryl derivatives may be represented by compounds like benzaldehyde propylene glycol acetal or 3-phenyl-2-furancarboxaldehyde.			
12. Is it hetero*aromatic* (B)? This question separates the aromatic heterocyclics for the purpose of considering whether they are polynuclear (question 14) or unsubstituted (question 13).	22	13	 NO (22)  YES (13)
13. Does the ring bear any substituents?	III	14	 NO (Class III)  YES (14)
14. Does the structure contain more than one *aromatic* (B) ring?	22	15	 NO (22)  YES (15)
15. Is it *readily hydrolysed* (H) to mononuclear residues? (If yes, treat the mononuclear heterocyclic residues by question 22 and any carbocyclic residue by question 16.)	33	22	 NO (33)  YES (22 & 16)
16. Is it a *common terpene* (D)-hydrocarbon, -alcohol, -aldehyde or -carboxylic acid (not a ketone)? Questions 16 and 17 deal with terpenes. A hydrocarbon terpene that is a *common terpene* (D) and has not already been put in class I by question 5, would go into class I by question 16.	17	I	 NO (17)  YES (Class I)
17. Is the substance *readily hydrolysed* (H) to a *common terpene* (D), -alcohol, -aldehyde or -carboxylic acid? (If the answer is yes, treat the hydrolysed residues separately and proceed to 18 for the terpene moiety and to 19 for any non-terpenoid moiety.) Since there may be substances that are hydrolysed to two or more residues, one of which is a terpene, treat the residues separately from question 18 onward to conclusion.	19	18	 NO (19)  YES (18 & 19)
18. Is the substance one of the following: (a) a vicinal diketone; or a ketone or ketal of a ketone attached to a terminal vinyl group (b) a secondary alcohol or ester of a secondary alcohol attached to a terminal vinyl group (c) allyl alcohol or its acetal, ketal or ester derivative (d) allyl mercaptan, an allyl sulphide, an allyl thioester or allyl amine (e) acrolein, a methacrolein or their acetals (f) acrylic or methacrylic acid (g) an acetylenic compound (h) an acyclic *aliphatic* (A) ketone, ketal or ketoalcohol with no other functional groups and with four or more carbons on either side of the keto group (i) a substance in which the *functional groups* (E) are all *sterically hindered* (J).	I	II	 NO (Class I)  YES (Class II)

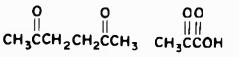
	If 'no' ....proceed to....	If 'yes' ....proceed to....	Examples with answers

Question 18 examines the terpenes (and later the open-chain and mononuclear substances by reference) to determine whether they contain certain structural features generally thought to be associated with some enhanced toxicity.

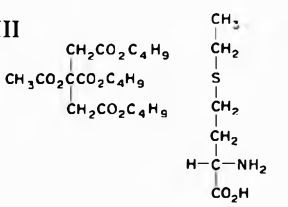
19. Is the substance *open chain* (G)?
Questions 19–21 deal with open-chain substances.

23 | 20 |  NO (23) YES (20)

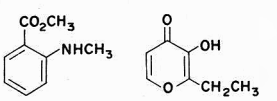
20. Is the structure a linear or *simply branched* (I) *aliphatic* (A) compound containing any one or combination of only the following *functional groups* (E): (a) four or less, each, of alcohol, aldehyde, carboxylic acid or esters and/or (b) one each of one or more of the following: acetal, either ketone or ketal but not both, mercaptan, sulphide (mono- or poly-), thioester, polyoxyethylene [(—OCH_2CH_2—)$_x$ with x no greater than 4], or primary or tertiary amine?
This question should be answered 'yes' if the structure contains one or any possible combination of alcoholic, aldehydic or carboxylic acid or ester groups, provided there are no more than four of any one kind. It should be answered 'yes' if the structure contains in addition to, or instead of, those just listed, any assortment of no more than one each of the following: acetal, either ketone or ketal but not both, mercaptan, mono- or polysulphide, thioester, polyoxyethylene, primary or tertiary amine. Answer the question 'no' if the structure contains more than four of any of the first set of groups, more than one of the second set, or any substituent not listed.

22 | 21 |  NO (22) YES (21)

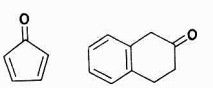
21. Does the structure contain three or more different types of functional groups (exclude methoxy and consider acids and esters as one functional type)?
Aliphatic (A) compounds containing three or more different functional groups (excluding methoxy) are too complex to permit satisfactory prediction of toxicity. They should go, therefore, into class III. However, we do not wish to put into class III polyesters and similar substances, so these and the methoxy compounds get passed along to question 18.

18 | III |  NO (18) YES (Class III)

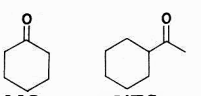
22. Is the substance a *common component of food* (C) or *structurally closely related* (K) to a *common component of food* (C)?
This question places in class II the natural, nature-identical and nearly nature-identical substances not already put into class I by physiological occurrence or structural criteria. An artificial (i.e. non-nature-identical) substance, or one not closely related, goes to question 33.

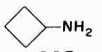
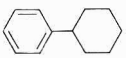
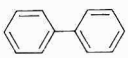
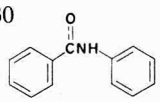
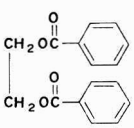
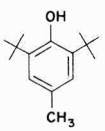
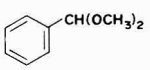
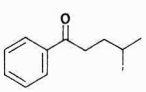
33 | II |  NO (33) YES (Class II)

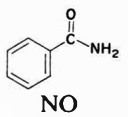
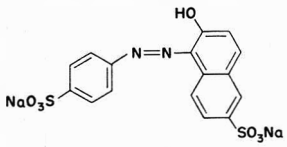
23. Is the substance *aromatic* (B)?
Questions 23–26 deal with alicylic substances.

24 | 27 |  NO (24) YES (27)

24. Is the substance monocarbocyclic (excluding cyclopropane or cyclobutane and their derivatives) with ring or *aliphatic* (A) side chains, unsubstituted or containing only alcohol, aldehyde, side-chain ketone, acid, ester, or sodium, potassium or calcium sulphonate or sulphamate, or acyclic acetal or ketal?

25 | 18 |  NO (25) YES (18)

	If 'no'	If 'yes'	Examples with answers
	....proceed to....		
25. Is the substance (a) a cyclopropane or cyclobutane with only the substituents mentioned in question 24 or (b) a mono- or bicyclic sulphide or mercaptan?	26	II	 NO (26)  YES (Class II)
26. Does the structure contain no functional groups other than those listed in question 24 and is it either a monocyloalkanone or a bicyclic compound with or without a ring ketone?	22	II	 NO (22)  YES (Class II)
27. Does (do) the ring(s) have any substituents? Questions 27–31 deal with aromatic compounds.	III	28	 NO (Class III)  YES (28)
28. Does the structure contain more than one *aromatic* (B) ring?	30	29	 NO (30)  YES (29)
29. Is it *readily hydrolysed* (H) to mononuclear residues? (If yes treat the individual aromatic mononuclear residues by question 30 and any other residue by question 19.)	33	30	 NO (33)  YES (30 & 19)
30. Disregarding ring hydroxy or methoxy does the ring bear substituents *other* than 1–5-carbon *aliphatic* (A) groups, either hydrocarbon or containing alcohol, ketone, aldehyde, carboxyl or simple esters that may be hydrolysed to ring substituents of five or less carbons? (If a simple ester that may be hydrolysed, treat the aromatic portion by question 18 and the residue by question 19.) This should be answered 'no' if the ring bears only aliphatic groups of five carbons or less, which are either hydrocarbon in nature or contain the groups listed. If the ring bears any other substituents than those listed, the question should be answered 'yes' and one should proceed to question 31.	18	31	 NO (18)  YES (31)
31. Is the substance an acyclic acetal, -ketal or -ester of any of the above substances (see question 30)? (If yes, assume hydrolysis and treat the non-aromatic residues by question 19 and the aromatic residue by question 18.) This question is simply designed to see whether the substance would fit within the definition of question 30 if it were not an acetal, a ketal or an ester. In other words, would the substance carry only the groups listed in question 30.	32	18	 NO (32)  YES (18)
32. Does the substance contain only the *functional groups* (E) listed in question 30, or their derivatives listed in question 31, but with any or all of the following: (a) a single fused non-aromatic carbocyclic ring, (b) *aliphatic* (A) substituent chains longer than five carbon atoms, or (c) a polyoxyethylene [(—OCH_2CH_2—)$_x$ with x no greater than 4] chain either on the aromatic ring or on an *aliphatic* (A) side chain? Part (a) is intended to allow simple derivatives of tetralin into class II while putting polycyclic compounds such as the steroids ultimately into class III except those that may be normal food	22	II	 NO (22)  YES (Class II)

	If 'no'	If 'yes'	Examples with answers
	....proceed to....		
components. Part (b) allows compounds with permitted functional groups but longer side chains into class II instead of sending them eventually into class III. Part (c) puts short-chain polyoxyethylene derivatives of aryl compounds into class II rather than class III.			
33. Does the substance bear on every major structural component at least one sodium, potassium, or calcium sulphonate or sulphamate for every 20 or fewer carbon atoms without any free primary amines except those adjacent to the sulphonate or sulphamate. Sodium, potassium, and calcium sulphonate and sulphamate salts have a strong tendency to decrease toxicity by promoting solubility and rapid excretion. This is particularly noticeable, for example, with some of the food colourings. It is important that the substance bears sufficient sulphonate groups, including one on each of the major structural fragments into which the original compound might be metabolized. This question serves to steer sulphonated compounds except those with amines non-adjacent to the sulphonate into a presumptively less toxic classification than the compounds would occupy if unsulphonated.	III	I	 NO (Class III)  YES (Class I)

Discussion

The decision tree

The questions that comprise the decision tree are a conscious compromise between discrimination and complexity. It will doubtless occur to many readers to suggest certain additional questions or changes of wording which would permit refinement and a clearer separation between the three classes. At the same time, we have tried to keep the questions as simple and few in number as possible. Further improvement is undoubtedly possible, and we welcome suggestions to that end.

We wish it were possible to make this whole effort more simple, but the task involves many simultaneous considerations which render it inherently complex. Undoubtedly we have not yet found ideally clear wording for many of the questions, but this represents the result of extensive trials by many workers.

Those who wish to use the decision tree should not be deterred by its initial complexity or by the slowness of the first attempts to use it. We have found that, with only a little training, equivalent to a careful reading of this paper, and a short period of practice, anyone with a background of moderate organic or biochemical knowledge can apply the decision tree with few errors and little difficulty. Reliability is enhanced when two or more individuals use it separately and compare their results. In practice, the tree contains a fair amount of redundancy. We have observed that in most instances an 'incorrect' answer leads, nevertheless, to the proper final classification. It should be stressed again that the decision tree is not intended to be used alone. It must be combined with information on present or probable intakes covered later in this discussion for an estimate of the implied hazard to be developed.

We have attempted several general tests of the utility of this decision tree. First, we have sought to classify, regardless of the availability of detailed toxicological data, a number of substances, some relatively innocuous and others highly toxic, to see if they fell into the appropriate classes. A host of substances known or presumed to be quite innocuous fell into class I. As far as we have been able to test, the recognized carcinogens, natural toxicants and pharmacologically potent drugs fall, as they should, in class III.

We are grateful to Dr. Marvin Legator and Mr. Stephen Rinkus for providing us with a list of 247 substances reported to cause cancer in two or more species. This was compiled from The Registry of Toxic Effects of Chemical Substances (Department of Health, Education, and Welfare, 1976). Twenty of these substances were polymers or inorganic materials to which this approach is not applicable. Of the remaining 227, all fall into class III with the single exception of xanthine, which falls into class I. Xanthine is a normal purine metabolite and an endogenous constituent of many human and animal tissues. The tests in which it has been reported to produce neoplasms involved not the oral route but subcutaneous administration and implantation. Thus we do not regard its classification as an aberrant result of this decision tree.

We have also applied a more critical evaluation by tabulating by class (I, II or III) the no-observed-adverse-effect levels (hereafter termed no-effect levels) for a number of food additives, drugs, industrial chemicals and pesticides. We have used the no-effect levels derived from relevant recent literature based on either short-term or chronic studies. Where these levels were reported in terms of dietary concentration, we used commonly accepted factors to convert to mg/kg body weight. In virtually all cases except those substances with no-effect levels above 500 mg/kg body weight/day, we have restricted the tabulation to toxicity tests in which the next higher feeding level above the no-effect level (i.e. the lowest level to show some adverse effect) was no more than five times the no-effect level. While this restraint rules out much of the

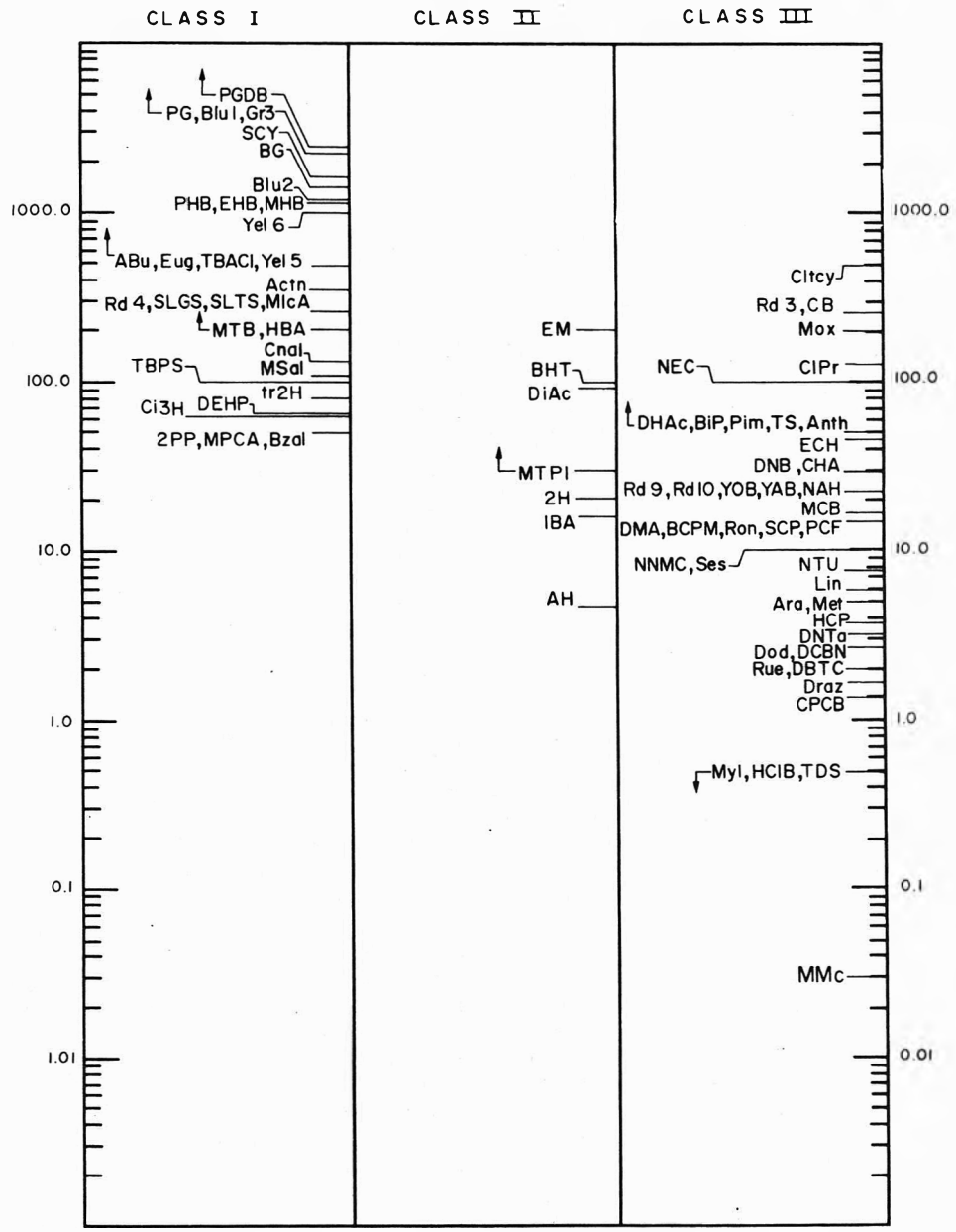


Fig. 2. Rank ordering of no-observed-adverse-effect levels within each class. Arrows indicate no-effect-levels greater or less than those shown; abbreviations refer to the substances listed in Table 1.

available toxicological data, it removes 'fuzziness' and provides a cleaner separation between classes than would otherwise occur.

With minor exceptions we have included without editing all data that have so far come to our attention, and that meet our criteria. Exceptions involved essentially duplicative data, one instance of the use of too few animals, and two cases in which the authors had chosen a lower no-effect level largely because even that provided an ample margin of safety.

The substances were run through the decision tree to determine the class into which they fell and then ranked by no-effect level within each class. The results of this tabulation are shown in Fig. 2 and Appendix 1 (p. 267). The appendix and the figure can be cross-related by the compound abbreviations indicated. Appendix 1 lists the abbreviation, common name, no-effect level and literature reference for the no-effect level. For the convenience of those who may wish to check their own use of the decision tree, the appendix also shows the 'track' for each substance through the tree.

It has been our general intent that the most toxic substance in class I (i.e. that with the lowest no-effect level) should have a no-effect level at or above 50 mg/kg body weight/day. With a hundredfold safety factor, this corresponds approximately to an intake of 25 mg/day by a human adult—a fairly substantial intake. Few exogenous chemicals that are not nutrients are consumed in larger amounts. It may well be that substances we have not sought out from the literature and plotted would fall into class I below

this level. This would raise the question of whether we wished to accept that state of affairs or, more probably, revise the decision tree structure so as to reclassify them into class II or class III.

Two facts stand out from the tabulation in Fig. 2. The first is the fact that the decision tree does result in a fairly clear-cut separation. All of the really innocuous substances fall into class I. All of the highly toxic compounds are in class III. Class II is both in between and gratifyingly smaller. We should be concerned if most substances wound up indecisively in class II.

The second obvious conclusion is that there is a slight overlap between the classes, particularly in the 10–100 mg/kg body weight area. This may seem to be a disadvantage, but on reflection, it is clear that this must be so. Substances much more innocuous than 1000 mg/kg body weight/day are so patently harmless that it is rare to find them adequately studied to the point of establishing no-effect levels. Similarly, substances much more toxic than 1 mg/kg body weight/day are so toxic that unless they are useful economic poisons, drugs or chemical warfare agents, they are unlikely to be used, and the data on them are unlikely to be published. Thus, the area roughly between 10 and 100 mg and to some extent on either side of it, is simply where the data base lies. These are the substances that are worth testing for reasons of utility and that require testing for reasons of probable hazard. Furthermore, inspection of the class III substances with no-effect levels of 50 mg/kg body weight or more indicates that virtually none of them could be judged in advance to be innocuous. They are 'unexpectedly nontoxic', and therefore merit retention in class III.

Estimates of presumable risk

To obtain an estimate of presumable risk, for setting priorities and for the preliminary establishment of criteria, one may combine in a grid, the estimate of toxic threat from the decision tree with data on intake (Table 1). We have utilized here daily per capita intake, mindful of its limitations and of the desirability—even in preliminary estimates—of taking into account the range of intakes across the population (Hall, Kahan, Merwin, Wharton, Dodger & Abrams, 1978).

In every box under each intake bracket for classes I and II is a figure derived from the presumptive, but conservative, no-effect level using the most toxic (lowest no-effect) level in that class (50 mg/kg for class I and 5 mg/kg for class III: see Fig. 2 and Appendix 1). That 'presumptive no-effect level' is divided by the upper limit of intake for that bracket, to obtain what we shall call the 'protection index' (PI). For example, the lowest no-effect level for any class I substance is 50 mg/kg body weight, equivalent to an intake of 2500 mg for a 50-kg adult; if the *per capita* daily intake of such a substance is between 1 and 10 μg (10^{-3}–10^{-2} mg) dividing 2500 mg by the top of that intake bracket (10 μg) gives a PI of 250,000 (Table 1). The other figures for classes I and II are similarly derived. No such figure can be adduced for class III, since there is not necessarily any definable lower limit of effect for this class. If we were to assume, however, that few if any ingested substances would have a no-effect level lower than 0·1 mg/kg equivalent to an intake of 5 mg/day, an intake of 0·1 μg/day would imply a PI of approximately 50,000. In this grid, the substances identified, on the basis of these presumptions as most hazardous (those with the lowest PI) are in the lower right and those identified as most safe are in the upper left.

The reader should note that we do not suggest that the actual 'safety factor' for a particular class I substance consumed at a designated intake is the same as the PI shown in Table 1. This is most obviously true at the higher intakes and higher presumptive toxicities. Only actual testing will establish a no-effect level from which a 'safety factor' can be deduced. The distribution of values within the different classes in Appendix 1 suggests that the experimentally determined no-effect level and the resulting safety factor will usually be at least an order of magnitude higher than the lower limit for the class assumed in Table 1.

We do not, then, assume that these PI values are accurate; indeed they are probably quite conservative. But they do provide an 'index of suspicion', by which to focus attention in an orderly way on the apparently greater hazards and to consign to low priority the very remote risks. Moreover, they provide a basis for a preliminary specification of the kinds of information appropriate for a demonstration of safety for a substance with that priority. We suggest four such categories, designated A–D.

The letter 'A' applied to PI values of 250,000 and higher represents the lowest priority. Unless other considerations intrude, priority A requires physical

Table 1. *Classification by presumable risk showing 'protection index' (PI) and categories of safety*

Class	PI* and category of safety for a daily per capita intake (in mg) of							
	$<10^{-5}$	10^{-5}–10^{-4}	10^{-4}–10^{-3}	10^{-3}–10^{-2}	10^{-2}–10^{-1}	0·1–1·0	1·0–10	>10
I	>250,000,000 A	>25,000,000 A	>2,500,000 A	>250,000 A	>25,000 B	>2500 C	>250 C	<250 D
II	>25,000,000 A	>2,500,000 A	>250,000 A	>25,000 B	>2500 C	>250 C	>25 D	<25 D
III	(500,000) A	(50,000) B	(5000) C	(500) C	(50) D	(?) D	(?) D	(?) D

*PI = $\frac{\text{lowest no-effect level for class (mg/kg body weight)} \times 50\text{ (kg body weight)}}{\text{maximum of intake range (mg) in column}}$.

and chemical data for each substance, but denotes no present need for the acquisition of actual animal data, at least for direct and most indirect additives. Certain types of simple screening tests may be applied when and if their reproducibility and correlation with feeding studies are adequately validated by sufficient collaborative testing on a broad variety of food constituents and contaminants.

'B' and 'C' represent progressively higher priority for more intensive investigation. While a variety of trade-offs may be contemplated in these intermediate categories among various kinds and quantities of data, both imply the need for animal data, and 'C' would ordinarily imply at least sub-chronic data plus other evidence of safe metabolic disposition. 'D' denotes the necessity for chronic studies, plus, where appropriate, other supporting data.

It should be evident that other factors beyond those dwelt on here affect the direction and extent of testing. The importance (irreplaceability) of a substance, the nature of the anticipated toxic effect, the level of organizational or social concern, the sensitivity of particular groups of users, more refined data on the distribution of exposure and, not least, the 'gut feeling' of the professional toxicologist must be involved. These additional factors should be of greater influence with respect to class III substances, where the PI is less clear and the anticipated adverse effects may range more widely and be more serious. The structure we suggest here is a guideline, not a channel. Even more importantly, we stress again that this is intended only as a guide to the acquisition of data, not as a substitute for data. When actual tests results are available they must weigh heavily in determining the direction and extent of further testing.

Finally, this procedure requires knowledge of chemical structure and reasonably accurate estimates of intake. Where either is lacking, this method of estimating hazard *must not* be applied. If structure is unknown, the decision tree cannot be used, and one must not assume that a substance of unknown structure is necessarily in class III. If intake is unknown, a most critical factor in determining hazard is absent. One cannot use the lower limit of sensitivity of an analytical method as the basis for an assumed intake in this approach. The method cannot be applied where positive analytical results or estimates of intake based on actual use in food are lacking.

We welcome application, criticism and improvement of the 'decision tree' as a tool, and of this approach to establishing priorities and criteria. We venture to believe that it has some general usefulness in helping to make a number of the thousands of decisions that lie ahead.

Acknowledgements—The preparation of this paper has been made possible only through the advice and assistance of a remarkably large number of people. Dr. Bernard L. Oser has provided encouragement, insight and suggestions, as have Drs Lauren A. Woods, Howard C. Spencer, David W. Fassett, Anthony M. Ambrose and R. Tecwyn Williams. Drs Otho D. Easterday and John C. Kirschman have given every possible assistance. Drs John A. Zapp, Jr., Robert A. Scala, Jessie M. Norris and John P. Frawley and Mr. Carrol S. Weil have been generous with their time and thought. Dr. Jan Stofberg and members of the Food Additives Committee of the Flavor & Extract Manufacturers' Association rendered invaluable assistance in proving and improving the decision tree by processing through it hundreds of different substances. Drs William G. Galetto and William N. Zeiger, Mr. Earl J. Merwin and Mrs. Merle I. Eiss did most of the literature search for toxicological data and provided the necessary structural information, including some corrections to the literature, from their formidable knowledge of organic nomenclature. Finally, many others in government, academia and industry have provided encouragement and advice.

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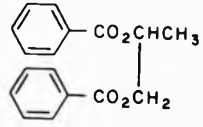
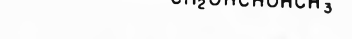
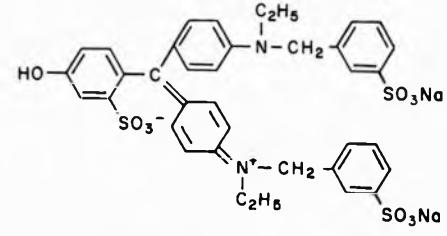
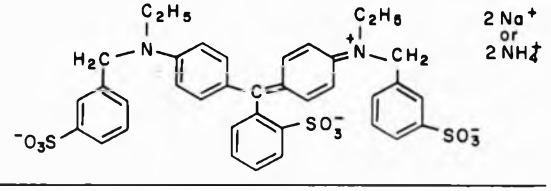
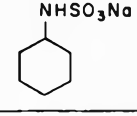
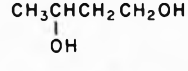
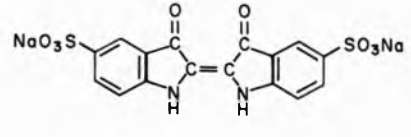
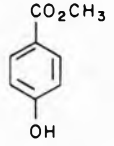
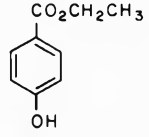
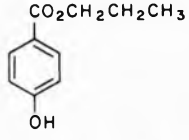
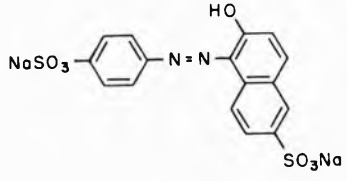
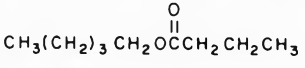
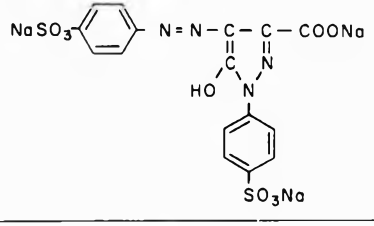
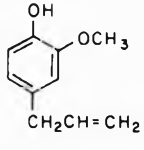
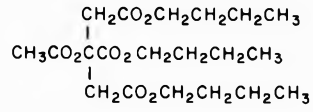
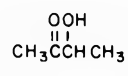
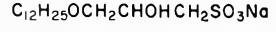
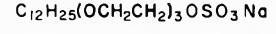
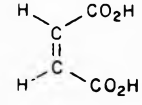
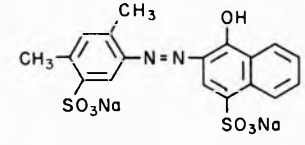
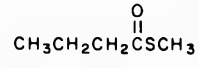
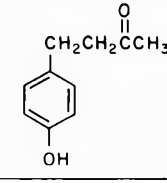
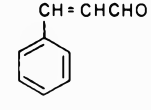
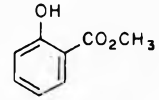
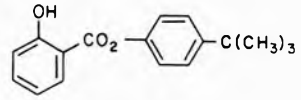
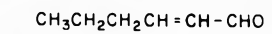
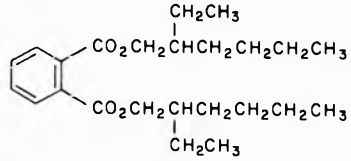
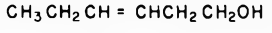
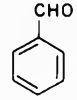
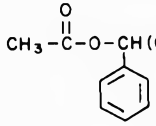
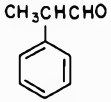
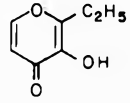
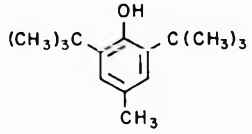
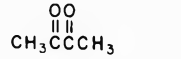
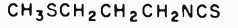
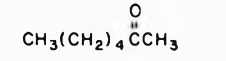
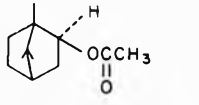
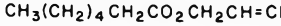
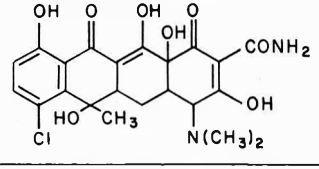
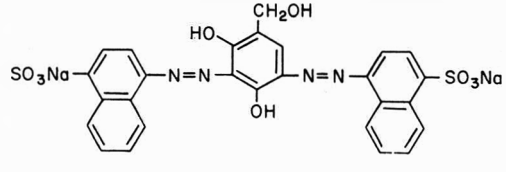
Appendix 1. *Tabulation of substances by class in order of decreasing no-observed-adverse-effect level*

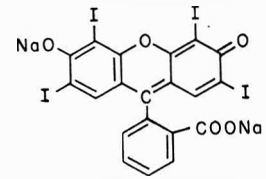
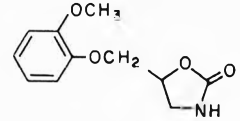
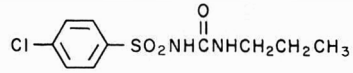
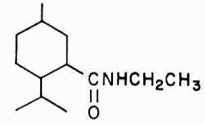
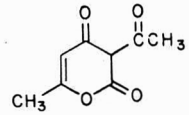
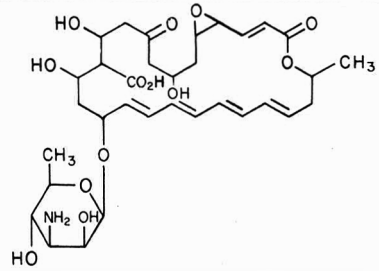
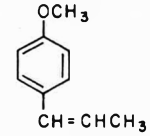
Abbreviation	Compound and track through decision tree (with reference to NEL in parenthesis)	No-effect level (mg/kg body weight)	Structure
	CLASS I		
PGDB	propylene glycol dibenzoate 1N, 2N, 3N, 5N, 6N, 7N, 16N, 17N, 19N, 23Y, 27Y, 28Y, 29Y, $^{19Y,20Y,21N,18N-I}_{30N,18N-I}$ (GRAS submission)	>2541	
PG	Propylene glycol 1N, 2N, 3N, 5N, 6N, 7N, 16N, 17N, 19Y, 20Y, 21N, 18N—I Gaunt *et al. Fd Cosmet. Toxicol.* 1972, **10**, 151)	>2500	
Gr3	FD & C Green No. 3 1N, 2N, 3Y, 4Y, 7N, 16N, 17N, 19N, 23Y, 27Y, 28Y, 29N, 33Y—I (Hansen *et al. Fd Cosmet. Toxicol.* 1966, **4**, 389)	2500	
Blu1	FD & C Blue No.1 1N, 2N, 3Y, 4Y, 7N, 16N, 17N, 19N, 23Y, 27Y, 28Y, 29N, 33Y—I (Hansen *et al. Toxic. appl. Pharmac.* 1966, **8**, 29)	2500	
SCY	Sodium cyclamate 1N, 2N, 3Y, 4Y, 7N, 16N, 17N, 19N, 23N, 24Y, 18N—I (Brantom *et al. Fd Cosmet. Toxicol.* 1973, **11**, 735)	1750	
BG	1,3-Butylene glycol 1N, 2N, 3N, 5N, 6N, 7N, 16N, 17N, 19Y, 20Y, 21N, 18N—I (Scala *et al. Toxic. appl. Pharmac.* 1964, **6**, 358)	1500	
Blu2	FD & C Blue No. 2 (Indigo carmine) 1N, 2N, 3Y, 4Y, 7Y, 8N, 10N, 11Y, 33Y—I (Gaunt *et al. Fd Cosmet. Toxicol.* 1969, **7**, 17)	1350	

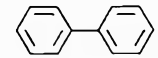
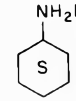
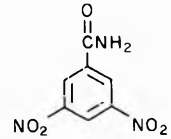
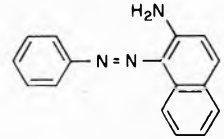
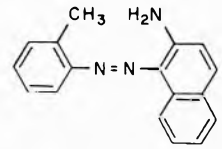
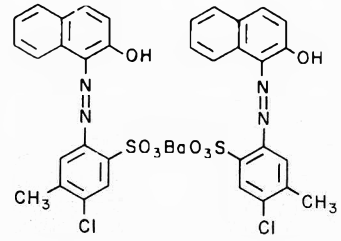
MHB	Methyl *p*-hydroxybenzoate 1N, 2N, 3N, 5N, 6N, 7N, 16N, 17N, 19N, 23Y, 27Y, 28N, 30N, 18N—I (Matthews *et al. J. Am. pharm. Ass.* 1956, **45**, 260)	1200	
EHB	Ethyl *p*-hydroxybenzoate 1N, 2N, 3N, 5N, 6N, 7N, 16N, 17N, 19N, 23Y, 27Y, 28N, 30N, 18N—I (Matthews *et al. J. Am. pharm. Ass.* 1956, **45**, 260)	1200	
PHB	Propyl *p*-hydroxybenzoate 1N, 2N, 3N, 5N, 6N, 7N, 16N, 17N, 19N, 23Y, 27Y, 28N, 30N, 18N—I (Matthews *et al. J. Am. pharm. Ass.* 1956, **45**, 260)	1200	
Yel6	FD & C Yellow No. 6 1N, 2N, 3Y, 4Y, 7N, 16N, 17N, 19N, 23Y, 27Y, 28Y, 29N, 33Y—I (W. H. Hansen, unpublished data; cited from *Interbureau By-Lines* 1962, **3**, no. 3)	1000	
ABu	Amyl butyrate 1N, 2N, 3N, 5N, 6N, 7N, 16N, 17N, 19Y, 20Y, 21N, 18N—I (Hagan *et al. Fd Cosmet. Toxicol.* 1967, **5**, 141)	> 500	
Yel5	FD & C Yellow No. 5 1N, 2N, 3Y, 4Y, 7Y, 8N, 10N, 11Y, 33Y—I (Davis *et al. Toxic. appl. Pharmac.* 1964, 6, 621)	500	
Eug	Eugenol 1N, 2N, 3N, 5N, 6N, 7N, 16N, 17N, 19N, 23Y, 27Y, 28N, 30N, 18N—I (Hagan *et al. Fd Cosmet. Toxicol.* 1967, **5**, 141)	500	

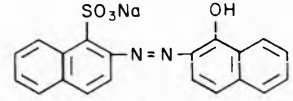
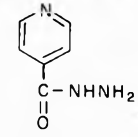
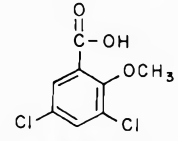
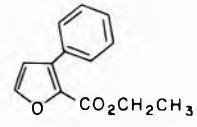
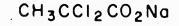
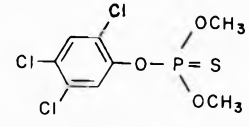
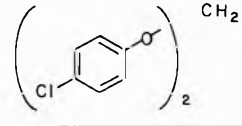
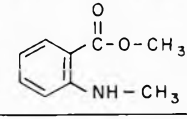
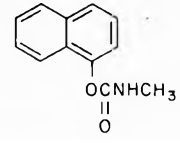
Abbreviation	Compound, track and reference	NEL (mg/kg)	Structure
TBACi	Tributyl acetylcitrate 1N, 2N, 3N, 5N, 6N, 7N, 16N, 17N, 19Y, 20Y, 21N, 18N—I (Finkelstein & Gold, *Toxic. appl. Pharmac.* 1959, **1**, 283)	500	
Actn	Acetoin 1N, 2N, 3N, 5N, 6N, 7N, 16N, 17N, 19Y, 20Y, 21N, 18N—I (Gaunt *et al. Fd Cosmet. Toxicol.* 1972, **10**, 131)	330	
SLGS	Sodium lauryl glyceryl ether sulphonate 1N, 2N, 3Y, 4Y, 7N, 16N, 17N, 19Y, 20N, 22N, 33Y—I (Tusing *et al. Toxic. appl, Pharmac.* 1962, **4**, 402)	250	
SLTS	Sodium lauryl trioxyethylene sulphate 1N, 2N, 3Y, 4Y, 7N, 16N, 17N, 19Y, 20Y, 21N, 18N—I (Tusing *et al. Toxic. appl. Pharmac.* 1962, **4**, 402)	250	
MlcA	Maleic acid 1N, 2N, 3N, 5N, 6N, 7N, 16N, 17N, 19Y, 20Y, 21N, 18N—I (Fitzhugh & Nelson, *J. Am. pharm. Ass.* 1947, **36**, 217)	250	
Rd4	FD & C Red No. 4 1N, 2N, 3Y, 4Y, 7N, 16N, 17N, 19N, 23Y, 27Y, 28Y, 29N, 33Y—I (Davis *et al. Toxic. appl. Pharmac.* 1966, **8**, 306)	250	
MTB	Methyl thiobutyrate 1N, 2N, 3N, 5N, 6N, 7N, 16N, 17N, 19Y, 20Y, 21N, 18N—I (GRAS submission)	>200	
HBA	*p*-Hydroxybenzyl acetone 1N, 2N, 3N, 5N, 6N, 7N, 16N, 17N, 19N, 23Y, 27Y, 28N, 30N, 18N—I (Gaunt *et al. Fd Cosmet. Toxicol.* 1970, **8**, 349)	200	
Cnal	Cinnamaldehyde 1N, 2N, 3N, 5N, 6N, 7N, 16N, 17N, 19N, 23Y, 27Y, 28N, 30N, 18N—I (Hagan *et al. Fd Cosmet. Toxicol.* 1967, **5**, 141)	125	

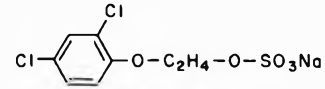
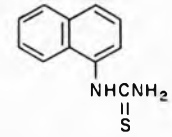
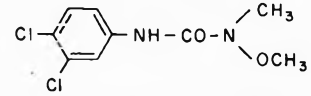
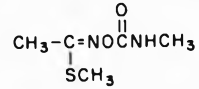
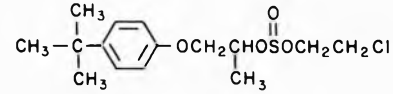
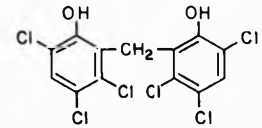
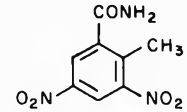
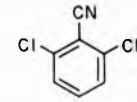
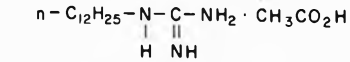
MSal	Methyl salicylate 1N, 2N, 3N, 5N, 6N, 7N, 16N, 17N, 19N, 23Y, 27Y, 28N, 30N, 18N—I (Packman *et al. Pharmacologist* 1961, **3**, 62)	105	
TBPS	*tert*-Butylphenyl salicylate 1N, 2N, 3N, 5N, 6N, 7N, 16N, 17N, 19N, 23Y, 27Y, 28Y, 29Y, 30N, 18N—I (Weil & McCollister, *J. agric. Fd Chem.* 1963, **11**, 486	100	
tr2H	*trans*-2-Hexenal 1N, 2N, 3N, 5N, 6N, 7N, 16N, 17N, 19Y, 20Y, 21N, 18N—I (Gaunt *et al. Fd Cosmet. Toxicol.* 1971, **9**, 775)	80	
DEHP	Di-(2-ethylhexyl) phthalate 1N, 2N, 3N, 5N, 6N, 7N, 16N, 17N, 19N, 23Y, 27Y, 28N, 30N, 18N—I (Weil & McCollister, *J. agric. Fd Chem.* 1963, **11**, 486)	65	
Ci3H	*cis*-3-Hexenol 1N, 2N, 3N, 5N, 6N, 7N, 16N, 17N, 19Y, 20Y, 21N, 18N—I (Gaunt *et al. Fd Cosmet. Toxicol.* 1969, **7**, 451)	62·5	
Bzal	Benzaldehyde 1N, 2N, 3N, 5N, 6N, 7N, 16N, 17N, 19N, 23Y, 27Y, 28N, 30N, 18N—I (Hagan *et al. Fd Cosmet. Toxicol.* 1967, **5**, 141)	50	
MPCA	Methylphenylcarbinyl acetate 1N, 2N, 3N, 5N, 6N, 7N, 16N, 17N, 19N, 23Y, 27Y, 28N, 30N, 18N—I (Gaunt *et al. Fd Cosmet. Toxicol.* 1974, **12**, 185)	50	
2PP	2-Phenylpropanal 1N, 2N, 3N, 5N, 6N, 7N, 16N, 17N, 19N, 23Y, 27Y, 28N, 30N, 18N—I (Pelling *et al. Fd Cosmet. Toxicol.* 1976, **14**, 249)	50	
	CLASS II		
EM	Ethyl maltol 1N, 2N, 3N, 5N, 6N, 7Y, 8N, 10N, 11N, 12N, 22Y—II (Gralla *et al. Toxic. appl. Pharmac.* 1969, **15**, 604)	200	

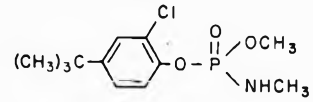
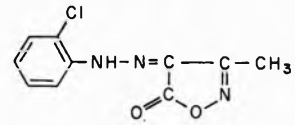
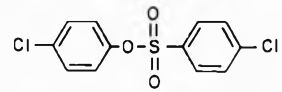
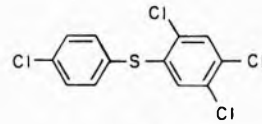
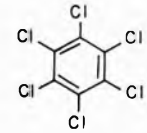
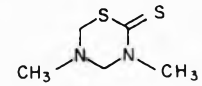
Abbreviation	Compound, track and reference	NEL (mg/kg)	Structure
BHT	Butylated hydroxytoluene 1N, 2N, 3N, 5N, 6N, 7N, 16N, 17N, 19N, 23Y, 27Y, 28N, 30N, 18Y—II (NTIS Publ. PB-221 202, GRAS Food Ingredients)	100	
DiAc	Diacetyl 1N, 2N, 3N, 5N, 6N, 7N, 16N, 17N, 19Y, 20N, 22Y—II (Colley *et al. Fd Cosmet. Toxicol.* 1969, **7**, 571)	90	
MTPI	3-Methylthiopropyl isothiocyanate 1N, 2N, 3N, 5N, 6N, 7N, 16N, 17N, 19Y, 20N, 22Y—II (GRAS submission)	>30	
2H	2-Heptanone 1N, 2N, 3N, 5N, 6N, 7N, 16N, 17N, 19Y, 20Y, 21N, 18Y—II (Gaunt *et al. Fd Cosmet. Toxicol.* 1972, **10**, 625)	20	
IBA	Isobornylacetate 1N, 2N, 3N, 5N, 6N, 7N, 16N, 17N, 19N, 23N, 24N, 25N, 26N, 22Y—II (Gaunt *et al. Fd Cosmet. Toxicol.* 1971, **9**, 355)	15	
AH	Allyl heptanoate 1N, 2N, 3N, 5N, 6N, 7N, 16N, 17N, 19Y, 20Y, 21N, 18Y—II (Hagan *et al. Toxic. appl. Pharmac.* 1965, **7**, 18; FDA File 997, 24 March 1964)	5	
	CLASS III		
Cltcy	Chlortetracycline 1N, 2N, 3Y, 4N—III (Dessau & Sullivan, *Toxic. appl. Pharmac.* 1961, **3**, 654)	500	
CB	Chocolate Brown HT 1N, 2N, 3Y, 4Y, 7N, 16N, 17N, 19N, 23Y, 27Y, 28Y, 29N, 33N—III (Hall *et al. Fd Cosmet. Toxicol.* 1966, **4**, 143)	250	

Rd3	FD & C Red No. 3 1N, 2N, 3Y, 4N—III (W. H. Hansen, unpublished data; cited from *Interbureau By-Lines* 1962, **3**, no. 3)	250	
Mox	Mephanoxalone 1N, 2N, 3N, 5N, 6N, 7Y, 8N, 10N, 11Y, 33N—III (Yeory *et al. Toxic. appl. Pharmac.* 1964, **6**, 642)	200	
ClPr	Chloropropamide 1N, 2N, 3Y, 4N—III (Delahunt *et al. Toxic. appl. Pharmac.* 1960, **2**, 195)	125	
NEC	*N*-Ethyl-2-isopropyl-5-methylcyclohexanecarboxamide 1N, 2N, 3N, 5N, 6N, 7N, 16N, 17N, 19N, 23N, 24N, 25N, 26N, 22N, 33N—III (GRAS submission)	100	
DHAc	Dehydroacetic acid 1N, 2N, 3N, 5N, 6N, 7Y, 8Y, 9N, 20N, 22N, 33N—III (Weil & McCollister, *J. agric. Fd Chem.* 1963, **11**, 486)	>50	
Pim	Pimaricin 1N, 2N, 3N, 5N, 6N, 7Y, 8N, 10Y—III (Levinskas *et al. Toxic. appl. Pharmac.* 1966, **8**, 97)	50	
Anth	Anethole 1N, 2N, 3N, 5N, 6Y—III (Joint FAO/WHO Expert Committee on Food Additives, *F.A.O. Nutr. Mtg Rep. Ser.* 1967, **44A**, 7; WHO/Food Add./68.33)	50	

Abbreviation	Compound, track and reference	NEL (mg/kg)	Structure
BiP	Biphenyl 1N, 2N, 3N, 5N, 6N, 7N, 16N, 17N, 19N, 23Y, 27Y, 28Y, 29N, 33N—III (Ambrose *et al. Fd Res.* 1960, **25,** 328)	50	
TS	Tin salts (organic) 1N, 2N, 3Y, 4N—III (de Groot *et al. Fd Cosmet. Toxicol.* 1973, **11,** 19)	50	$Sn^{+2}(C_4H_4O_6)^{-2}$ $Sn^{+2}(C_2O_4)^{-2}$
ECH	Ethylene chlorohydrin 1N, 2N, 3Y, 4N—III (Oser *et al. Fd Cosmet. Toxicol.* 1975, **13,** 313)	45	$ClCH_2CH_2OH$
CHA	Cyclohexylamine hydrochloride 1N, 2N, 3Y, 4Y, 7N, 16N, 17N, 19N, 23N, 24N, 25N, 26N, 22N, 33N—III (Gaunt *et al. Fd Cosmet. Toxicol.* 1976, **14,** 255)	30	
DNB	3,5-Dinitrobenzamide 1N, 2Y—III (Kerr *et al. Toxic. appl. Pharmac.* 1965, **7,** 488)	30	
YAB	Yellow AB (1-phenylazo-2-naphthylamine) 1N, 2N, 3N, 5N, 6N, 7N, 16N, 17N, 19N, 23Y, 27Y, 28Y, 29N, 33N—III (Hansen *et al. Toxic. appl. Pharmac.* 1963, **5,** 16)	25	
YOB	Yellow OB (1-*o*-tolylazo-2-naphthylamine) 1N, 2N, 3N, 5N, 6N, 7N, 16N, 17N, 19N, 23Y, 27Y, 28Y, 29N, 33N—III (Hansen *et al. Toxic. appl. Pharmac.* 1963, **5,** 16)	25	
Rd9	D & C Red No. 9 1N, 2N, 3Y, 4N—III (Davis & Fitzhugh, *Toxic. appl. Pharmac.* 1962, **4,** 200)	25	

Rd10	D & C Red No. 10 1N, 2N, 3Y, 4Y, 7N, 16N, 17N, 19N, 23Y, 27Y, 28Y, 29N, 33N—III (Davis & Fitzhugh, *Toxic. appl. Pharmac.* 1963, **5,** 728)	25	
NAH	Isonicotinic acid hydrazide 1N, 2N, 3N, 5N, 6N, 7Y, 8N, 10N, 11Y, 33N—III (Harper & Worden, *Toxic. appl. Pharmac.* 1966, **8,** 325)	25	
MCB	2-Methoxy-3,5-dichlorobenzoic acid 1N, 2N, 3Y, 4N—III (Edson & Sanderson, *Fd Cosmet. Toxicol.* 1965, **3,** 299)	16	
PCF	3-Phenyl-2-carbethoxyfuran 1N, 2N, 3N, 5N, 6N, 7Y, 8N, 10N, 11N, 12Y, 13Y, 14Y, 15N, 33N—III (GRAS submission)	15	
SCP	Sodium 2,2-dichloropropionate 1N, 2N, 3Y, 4N—III (Weil & McCollister, *J. agric. Fd Chem.* 1963, **11,** 486)	15	
Ron	Ronnel 1N, 2N, 3Y, 4N—III (McCollister *et al. J. agric. Fd Chem.* 1959, **10,** 689)	15	
BCPM	Bis-(*p*-chlorophenoxy)methane 1N, 2N, 3Y, 4N—III (Weil & McCollister, *J. agric. Fd Chem.* 1963, **11,** 486)	15	
DMA	Dimethyl anthranilate 1N, 2N, 3N, 5N, 6N, 7N, 16N, 17N, 19N, 23Y, 27Y, 28N, 30Y, 31N, 32N, 22N—III (Gaunt *et al. Fd Cosmet. Toxicol.* 1970, **8,** 359)	15	
NNMC	α-Naphthyl *N*-methylcarbamate 1N, 2N, 3N, 5N, 6N, 7N, 16N, 17N, 19N, 23Y, 27Y, 28Y, 29N, 33N—III (Weil & McCollister, *J. agric. Fd Chem.* 1963, **11,** 486)	10	

Abbreviation	Compound, track and reference	NEL (mg/kg)	Structure
Ses	Sesone 1N, 2N, 3Y, 4N—III (Carpenter *et al. J. agric Fd Chem.* 1961, **9,** 382)	10	
NTU	α-Naphthylthiourea 1N, 2N, 3N, 5N, 6N, 7N, 16N, 17N, 19N, 23Y, 27Y, 28Y, 29N, 33N—III (Fitzhugh, *Proc. Soc. exp. Biol. Med.* 1947, **64,** 305)	7·5	
Lin	Linuron 1N, 3N, 3Y, 4N—III (Hodge *et al. Fd Cosmet. Toxicol.* 1968, **6,** 171)	6	
Met	Methomyl 1N, 2N, 3N, 5N, 6N, 7N, 16N, 17N, 19Y, 20N, 22N, 33N—III (Kaplan & Sherman, *Toxic. appl. Pharmac.* 1977, **40,** 1)	5	
Ara	Aramite 1N, 2N, 3Y, 4N—III (Oser & Oser, *Toxic. appl. Pharmac.* 1962, **4,** 70)	5	
HCP	Hexachlorophene 1N, 2N, 3Y, 4N—III (Nakaue *et al. Toxic. appl. Pharmac.* 1973, **24,** 239)	3·7	
DNTa	3,5-Dinitro-*o*-toluamide 1N, 2Y—III (Weil & McCollister, *J. agric. Fd Chem.* 1963, **11,** 486)	3·1	
DCBN	2,6-Dichlorobenzonitrile 1N, 2N, 3Y, 4N—III (van Genderen & van Esch, *Fd Cosmet. Toxicol.* 1968, **6,** 261)	2·5	
Dod	Dodine 1N, 2N, 3N, 5N, 6N, 7N, 16N, 17N, 19Y, 20N, 22N, 33N—III (Levinskas *et al. Toxic. appl. Pharmac.* 1961, **3,** 127)	2·5	
DBTC	Di-*n*-butyltin dichloride 1N, 2N, 3Y, 4N—III (Gaunt *et al. Fd Cosmet. Toxicol.* 1968, **6,** 599)	2	

Rue	Ruelene 1N, 2N, 3Y, 4N—III (McCollister *et al. Fd Cosmet. Toxicol.* 1968, **6**, 185)	2	
Draz	Drazoxolon 1N, 2N, 3Y, 4N—III (Clark & McElligott, *Fd Cosmet. Toxicol.* 1969, **7**, 481)	1·5	
CPCB	*p*-Chlorophenyl-*p*-chlorobenzenesulphonate 1N, 2N, 3Y, 4N—III (McCollister *et al. Fd Cosmet. Toxicol.* 1964, **2**, 563)	1·25	
TDS	2,4,5,4′-Tetrachlorodiphenyl sulphide 1N, 2N, 3Y, 4N—III (Verschuuren *et al. Toxicology* 1973, **1**, 63)	0·5	
HClB	Hexachlorobenzene 1N, 3N, 3Y, 4N—III (Kuiper-Goodman *et al. Toxic. appl. Pharmac.* 1977, **40**, 529)	0·5	
Myl	Mylone 1N, 2N, 3N, 5N, 6N, 7Y, 8N, 10N, 11Y, 33N—III (Smyth *et al. Toxic. appl. Pharmac.* 1966, **9**, 521)	<0·5	
MMc	Methylmercuric chloride 1N, 2N, 3Y, 4N—III (Ikeda *et al. Toxicology* 1973, **1**, 361)	0·03	CH_3ClHg

Fd Cosmet. Toxicol. Vol. 16. pp. 277–281. Pergamon Press 1978. Printed in Great Britain

REVIEWS OF RECENT PUBLICATIONS

Second Task Force For Research Planning In Environmental Health Science. Human Health and the Environment—Some Research Needs. Report of the Second Task Force for Research Planning in Environmental Health Science, DHEW Publication No. NIH 77-1277. US Department of Health, Education, and Welfare, Washington, DC, 1977. pp. xxx + 498. $1.92 (from Superintendent of Documents, US Government Printing Office, Washington DC 20402).

An extensive survey of needs in environmental health research, intended to guide national efforts for the next 5–8 years, is presented in this report of the Second Task Force for Research Planning in Environmental Health Science. Successive chapters deal with the main types of pollutants and other environmental chemicals, methods of measuring and forecasting levels of human exposure, the investigation of biological mechanisms and testing for toxicity, carcinogenesis, mutagenesis and effects on reproduction and human development, behavioural toxicology, the systematic examination of organ systems for interaction with environmental toxicants, methods and resources for estimating disease risk in man, and professional and scientific education and the supply of suitably trained personnel.

The Task Force members responsible for a chapter concerned principally with food and water, recommend that in the field of food additives the full spectrum of the biological activities of nitrates, nitrites and nitrosamines should be elucidated, to determine whether further control measures are necessary, and research should be encouraged into substances that could be effective substitutes for the two preservatives or that might prevent their conversion to nitrosamines. Other topics considered worthy of further study are the effects of high intakes of sodium chloride and phosphates, especially during infancy, with emphasis on their respective effects on hypertension and bone growth. Because of the large numbers of food additives and the degree of population exposure, the group recommends that safety evaluations of all such substances should be re-examined and updated at frequent intervals. The interactions of important food additives with major dietary constituents such as methionine, the alterations of dietary components resulting from processing, and the possible toxic effects of derived products should be studied in detail, and procedures to prevent the accumulation of undesirable products in food should be investigated.

Consideration is given to heavy-metal contaminants, with the movement of mercury into the food chain and events leading to its alkylation being regarded as "critical" research areas. The extent to which food contributes to lead-related disease, the possible role of dietary cadmium in essential hypertension, and interactions with and exposure of the mammalian organism to copper, zinc, manganese, selenium and arsenic are also considered to require elucidation. Other contaminants considered are pesticides, for which specified research needs include the identification of sources of contamination and of those reactions with food constituents likely to lead to harmful effects in the consumer. Detailed recommendations on mycotoxins include a suggestion that mycotoxin contamination of water supplies should be investigated. Other impurities in drinking-water, particularly chlorinated hydrocarbons and products of ozonation, are also considered to merit further research.

A long and particularly interesting section recommends an organized approach to the examination of the adverse effects of environmental agents on specific organ systems, and points out that the promotion of such an approach would necessitate the acceleration of basic research on the fundamental processes taking place in each organ and the development of appropriate animal models for clarifying environmental disease processes. Another essential would be the direction of epidemiological investigations towards those diseases of the major organ systems currently of largely unknown aetiology.

Pesticide Residues in Food. Report of the 1975 Joint Meeting of the FAO Working Party of Experts and the WHO Expert Committee on Pesticide Residues. *Tech. Rep. Ser. Wld Hlth Org.* 1976, **592.** pp. 45. Sw.fr. 6.00 (available in UK through HMSO).

1975 Evaluations of Some Pesticide Residues in Food. The Monographs. *WHO Pestic. Residues Ser.* 1976, No. 5; Geneva, 1976. pp. 409. Sw.fr. 32.00 (available in UK through HMSO).

At the 1975 Joint Meeting of these groups, the data on 39 pesticides were reviewed with the aim of establishing or revising the ADI and/or the maximum residue limits recommended for specific foodstuffs. The first publication cited above contains a summary of the conclusions reached, together with discussions of some general problems and recommendations for future work. In the second volume are monographs, or addenda to existing monographs, reviewing the toxicological and residue data on 37 of these pesticides, two (ethephon and carbofuran) having been omitted because insufficient information was available to establish an ADI.

Of the pesticides evaluated for the first time, chlorpyrifos-methyl was allocated an ADI of 0·01 mg/kg, and temporary ADIs set pending the provision of further data were 0·005 mg/kg for cyanofenphos and 0·2 mg/kg for *sec*-butylamine. For pesticides previously evaluated, the ADIs set were 0·003 mg/kg for bromophos-ethyl, 0·002 mg/kg for disulfoton, 0·001 mg/kg for leptophos (temporary), 0·005 mg/kg for methidathion and 0·007 mg/kg for quintozene. A

previously-allocated ADI of 0·08 mg/kg for thiophanate-methyl was confirmed, and that for monocrotophos was increased to 0·0006 mg/kg. Temporary ADIs previously set for coumaphos, fenthion and omethoate (each of 0·0005 mg/kg), parathion-methyl (0·001 mg/kg) and chlordimeform (0·01 mg/kg) were extended, but that for trichlorphon was reduced to 0·005 mg/kg, pending the completion of a further carcinogenicity study to clarify conflicting findings.

Maximum residue limits in specific commodities were also set for the three newly-evaluated pesticides, and those for the reconsidered compounds and for benomyl, 2,4-D and demeton were reviewed in the light of new information. Also reconsidered from this point of view were a number of compounds referred from the 8th Session of the Codex Committee on Pesticide Residues, including aldrin/dieldrin, bromophos, carbaryl, chlorobenzilate, chlorpyrifos, cyhexatin, diazinon, endosulfan, endrin, ethion, heptachlor, lindane, malathion, 2-phenylphenol, phosalone and thiabendazole. Guideline levels (indicating the level of residues resulting from recommended uses) were published for bioresmethrin and methomyl, for which no ADIs could be established in the absence of toxicological data.

A general problem considered at the Meeting was the testing of pesticides for mutagenicity. As the methodology of such testing is undergoing rapid development, no particular test(s) could be recommended, but it was felt that mammalian test results should carry more weight than those in lower organisms or isolated cell systems. Positive results from microbial tests, unsupported by other information, were regarded as uninterpretable for the purposes of establishing an ADI. However, mutagenicity tests were considered particularly desirable for certain substances, such as those yielding metabolites with stable carbonium ions or of strong electrophilic reactivity. It was suggested that WHO should convene a meeting with the aim of recommending tests suitable for predicting human mutagenic hazards, and of considering the possibility of establishing no-effect levels for mutagenic action.

Delayed neurotoxicity, particularly that caused by leptophos, was another problem discussed at the Meeting. It was noted that man may be the most sensitive species in this respect, although adult hens, cats, dogs, calves and sheep are also susceptible. Fortunately the phenomenon appears to be dose-related, and an ADI may therefore be estimated on the basis of studies in susceptible animal species. Further work was considered desirable on possible mechanisms of action, and on the hazards of occupational exposure. Data on the occupational hazards of other pesticides were also regarded as important for evaluating the safety of residues in food, and it was recommended that such information should be made available for future discussions.

An illuminating exercise undertaken at the 1975 Meeting was a comparison of the potential daily intakes of pesticide residues with their ADIs. Food consumption data from five countries in three regions of the world were used to calculate the intake of 14 pesticides, assuming that all residues were at the maximum recommended limits in every foodstuff. It emerged that there was theoretically no possibility of exceeding the ADIs of bromophos-ethyl, chlordimeform, chlorpyrifos-methyl, cyanofenphos, 2,4-D, demeton, methidathion, *sec*-butylamine or trichlorfon, but those of disulfoton, leptophos, monocrotophos and parathion-methyl could be exceeded by up to four times in at least one country, and for fenthion and ADI could be exceeded in all five countries by some 6–15 times. The Meeting accordingly recommended further work on the occurrence and fate of residues of the last five pesticides, followed by total-diet studies if the ADIs still appeared to be exceeded.

Lead in Drinking Water. A Survey in Great Britain, 1975–1976. Report of an Interdepartmental Working Group. Department of the Environment Pollution Paper No. 12. HMSO, London, 1977. pp. ix + 47. £1.15.

The lead content of drinking-water put into public supply in Great Britain is below the limit of 0·1 mg/litre recommended by WHO (*Cited in F.C.T.* 1972, **10**, 228), normally by a large factor. However, in 1975 a limit of 0·05 mg lead/litre was proposed in an EEC draft directive, without any indication as to whether it should apply to the water as put into supply or as it leaves the tap (*Off. J. Europ. Commun.* 1975, **18** (C214), 2). This proposal prompted a nationwide survey of lead in tap-water, the results of which have now been published.

Of the 3181 households sampled over the country as a whole, 9·0% had lead concentrations exceeding 0·10 mg/litre in the first water drawn in the morning, and 20·4% had concentrations exceeding 0·05 mg/litre in this 'first draw' water. In water drawn later in the day, the lead concentrations exceeded 0·10 and 0·05 mg/litre in 4·3 and 10·3% of households, respectively. The situation was at its worst in Scotland, where the proportion of households exceeding 0·05 mg/litre in the 'first draw' water was as high as 40·6%, and levels in excess of 0·1 and 0·3 mg/litre were found in 27·6 and 11·4% of households, respectively. Even in 'daytime' samples, 34·4% of the Scottish households had more than 0·05 mg lead/litre, 21·0% exceeded 0·10 mg/litre and 5·0% exceeded 0·30 mg/litre. At the last level, the intake of lead from 1·5 litres water/day alone would exceed the FAO/WHO 'provisional tolerable weekly intake' for adults of 3 mg (*Cited in F.C.T.* 1974, **12**, 407). However, over the country as a whole only 0·9% of households exceeded 0·30 mg/litre in their daytime water samples and only a slightly higher proportion (1·6%) exceeded this level in 'first draw' water.

The higher levels of lead tended to occur in smaller households, where the inhabitants were all adult, and where the houses were built in 1944 or earlier. Not unexpectedly, the presence of lead or lead/copper piping and the use of long lengths of such piping also correlated with higher levels. More surprisingly, although higher concentrations tended to occur in acid waters, many alkaline waters also contained lead in excess of 0·05 mg/litre, suggesting that attempts to control plumbosolvency by adding alkali to soft waters may not necessarily improve the situation. A limited survey of blood-lead levels produced some indication that blood levels may rise as the water

levels of lead rise, but a full-scale epidemiological survey (now planned) is needed to confirm this finding. Four adults in Scotland with blood levels above 1·75 μmol/litre (regarded as the upper limit of normality) are also being investigated in more detail.

The survey showed that high levels of lead in water are more widespread than expected, and further exploratory work is being given urgent priority. This will include an evaluation of the contribution of water to the total absorption of lead from all sources in houses where the water supply contains the highest concentrations. However, no evidence emerged from this study of any general problem of chronic lead poisoning arising from the public water supply, and there were no indications that blood levels may exceed medically accepted limits except in an unusual combination of circumstances.

Environmental Health Criteria 2: Polychlorinated Biphenyls and Terphenyls. WHO Task Group. Published under the joint sponsorship of the United Nations Environment Programme and the World Health Organization; WHO, Geneva, 1976. pp. 85. Sw.fr. 10.00 (available in UK through HMSO).

Late in 1972 WHO convened a meeting in Geneva on environmental health criteria and standards. At the meeting it was pointed out that WHO gave high priority to the establishment of environmental health criteria and standards to protect human health from adverse environmental factors. These criteria were to be basic tools for action against pollution, for the planning of abatement programmes, for the establishment of national standards and for the evaluation of environmental control programmes. The role of WHO was to collect the available scientific information, to evaluate it and to assist governments in applying it to environmental health programmes.

The polychlorinated biphenyls (PCBs) and terphenyls (PCTs) are the second in a series of materials currently being examined within the above framework. Publications on original work form the basis of the document, although recent papers reviewing the environmental and health aspects of PCBs have also been used. Despite the fact that commercial production of PCBs began back in the 1930s, the dangerous implications of their widespread use were not fully recognized until serious cases of poisoning were reported both in man and animals. Such incidents have now ranked the PCBs in importance alongside environmental pollutants such as lead and mercury.

For those interested in making a rapid appraisal of the situation, the first few pages of this publication are set aside to provide a summary which reveals the main areas of interest and makes recommendations for future research. The remainder of the document presents a comprehensive account of the findings from which an evaluation of the environmental significance of the materials was made. This part consists of sections on chemical properties and analytical methods, the sources of environmental pollution, transport and transformation within the environment, the extent of human exposure, the metabolism of the compounds, animal toxicity studies and epidemiological and clinical studies of effects in man.

Despite the vast amount of information that has accumulated on the PCBs, the WHO Task Group was able to point out numerous areas where further information would be of use. Although the environmental significance of the PCBs is quite well defined, any assessment of no-detectable-effect levels is complicated by the different activities of the component PCBs and by the presence of impurities, such as the chlorinated dibenzofurans. In addition, the PCTs remain very much an unknown force.

The document provides, nevertheless, a valuable contribution towards our knowledge of these compounds, even though much work is still required for an adequate environmental appraisal. It is interesting to note that, even in the presence of these uncertainties, regulations have been drawn up to limit the residue of PCBs in foods and feedstuffs, and restrictions have been placed on the commercial exploitation of these materials in a number of countries.

Pesticide Residues in Tobacco and Tobacco Products. III. Pesticide Residues Found in Tobacco—Toxicological Aspects of Residues in Tobacco. Information on Agriculture Series No. 26. Commission of the European Communities. EEC, Brussels, 1976. pp. xi + 286. £3.10 (available in UK through HMSO).

This large report, prepared by a group of EEC experts, follows an earlier, general, report and a separate appendix, detailing plant-protection methods, legislation and methods of analysis, published last year as Nos 14 and 23 of the same series. The third volume consists of two further annexes to the main report, surveying the pesticide residues in tobacco of various origins (as determined by analyses conducted in 1969–75) and the risks that these residues present for the smoker.

In the first, pesticides have been divided into three categories, namely insecticides no longer recommended (DDT, six cyclodienes, BHC isomers and toxaphene), registered chemicals leaving residues higher than tolerances assigned for food of plant origin (11 dithiocarbamates and five herbicides) and registered chemicals leaving residues not exceeding tolerances fixed for food of plant origin (107 chemicals of all kinds). The first two categories are discussed in detail, while the third is dealt with in the form of alphabetically arranged tables. In the second annex, the toxicology of 46 pesticides is discussed largely in terms of percentage loss during curing and transfer to the smoke. Data on pyrolysis products, fate within the body and inhalation and skin painting studies are included when available. It is concluded that if any residue tolerances are proposed, these should apply to finished tobacco products rather than to the raw tobacco, and should be based on residues detected in the smoke rather than on tolerances that have already been established for food products.

Methods Used in Establishing Permissible Levels in Occupational Exposure to Harmful Agents. Report of a WHO Expert Committee with the participation of ILO. *Tech. Rep. Ser. Wld Hlth Org.* 1977, **601**, pp. 68. Sw.fr. 8.00 (available in UK through HMSO).

Permissible levels for occupational exposure to

harmful chemicals are a basic factor in the protection of workers' health in all developed industrial countries. We have previously observed that the recommendations and requirements of different authorities for the control of any one chemical agent diverge much more widely than would be expected on the basis of scientific data alone. The report cited above points out that most variability occurs in relation to the type of adverse response deemed to be relevant, the acceptable variation in the vulnerability of individuals within the working population and the extent to which an ill-defined effect should be allowed to continue. Perhaps the greatest imponderable is that of extrapolating data from animals to man, and it is the food toxicologist, rather than those concerned with inhalation and skin effects, who has most experience of this perennial problem. As a result, only in the former category has a clearly defined 'safety factor' become widely accepted. The actual size of the safety margin depends, of course, on many factors, including the number of animals and, preferably, of human volunteers in relation to the population at risk and the type of effect observed. Thus, a much larger margin of safety is required for liver toxicity than for eye irritation.

The report stresses that the monitoring of total exposure of workers to a chemical is achieved most accurately through biological sampling of blood, urine and exhaled air. This reflects uptake through the lungs, skin and gastro-intestinal tract and facilitates the detection of the early biochemical changes that precede adverse biological effects. At the same time, monitoring of workroom air is required to determine compliance with existing standards.

This latest WHO report is aimed particularly at the developing countries, where it is clearly important that permissible levels should be established and taken into account when new industrial plants are at the planning stage. Since pre-employment medical screening is rare, workers may be particularly vulnerable to the effects of chemicals, which are rendered more accessible to the body in areas of high temperature and humidity. Priority substances may be different from those in industrialized countries, and may include textile and wood dusts and dusts derived from other vegetable and from animal sources. In such cases, the report urges foreign industries operating in these developing countries to comply with strict occupational health and safety standards.

In order to define more clearly the relationship between exposure, uptake and biological response, the Committee recommends prospective epidemiological studies, particularly on the effects of metals, organic solvents and dusts, and fibrous mineral dusts. Other recommendations include the undertaking of experiments to monitor the effects of combined exposures in processes involving several chemical agents, and the effects of exposure on the reproductive system.

It is notable that the Committee recommends a hierarchial system of toxicity testing, beginning with short-term tests and progressing where necessary to long-term studies. It is suggested that short-term 'predictive' tests for mutagenicity may be validated by combination with other *in vitro* tests of biological fluids from workers exposed to the same agents and by assessment of cytological abnormalities in exposed workers.

For the toxicologist, this report contains little that has not been stated before in many other places. Its real value lies in alerting governments, occupational health institutes and, particularly, the multinational companies with responsibility for countless workers who currently lack adequate occupational health care.

Report of the Government Chemist 1976. Department of Industry: Laboratory of the Government Chemist. HMSO, London, 1977. pp. iv + 166. £3.00.

As in previous years, the analysis of animal fats for pesticide residues formed an important part of the Laboratory's work during 1976. Home-produced mutton fat sampled from mid-1975 showed an increasing tendency to contain above-normal levels of γ-BHC, and sometimes also of the α- and β-isomers, reflecting the use of sheep dips containing γ-BHC in compliance with the Sheep Scab (Dipping Area) (No. 2) Order, 1976. A small minority of UK beef-fat samples also contained raised levels of BHC isomers, particularly in the early part of the year, although the reason for this was uncertain. Occasional high levels of dieldrin in both mutton and beef fat similarly lacked explanation. New Zealand fats continued to show a predominance of DDT-derived residues, mostly in the form of *p,p'*-DDE, whereas BHC and dieldrin were prominent in veal fat and butter from EEC countries, together with low levels of hexachlorobenzene and heptachlor epoxide.

In the livers of wild birds, polychlorinated terphenyls were detected at maximum levels of up to 1·2 ppm, the levels being proportional to, but one or two orders lower than those of the polychlorinated biphenyls found in the same specimen. It was concluded that, at least in the six bird species investigated, the polychlorinated terphenyls did not contribute greatly to the total burden of organochlorine compounds.

An EEC Directive on the fixing of maximum permitted levels for undesirable substances and products in feedingstuffs debarred the use of Indian species of *Brassica* in all feeds. In connexion with Brussels negotiations on this point, the Laboratory studied the goitrogen content of 48 samples of Indian rape meal. The content of allyl, butenyl and pentenyl isothiocyanates was less than 3000, 10,000 and 200 ppm respectively in all samples, and 5-vinyloxazolidine-2-thione, where detected, did not exceed 100 ppm. However, the total isothiocyanate content for all the samples expressed as the allyl compound exceeded the general limit of 4000 ppm prescribed in the directive for rape cake or meal sold as a straight animal feed. High levels of 5-vinyloxazolidine-2-thione (up to 8700 ppm) were found in rape meal from Chile, Canada, Algeria and Germany, together with a low allyl isothiocyanate content and moderate levels of the other two isothiocyanates, whereas in Ethiopian rape meal the proportions of these substances were reversed.

In human foods the range of trace elements monitored under the total diet survey was further extended during the year to include antimony and tin. Levels

of arsenic, first included in 1975, were found to be unexceptional in beverages and in dairy, cereal and meat products, apart from meat offal. Potable water was also analysed for metals, including copper, which was found in an increasing number of samples at levels often exceeding 0·2 ppm. These samples were often derived from multistorey buildings, where long runs of copper piping have to be used. In only a few cases did the concentrations of lead and zinc give rise to concern, and, although a significant number of waters contained detectable levels of cadmium and arsenic, the concentrations were well below WHO and proposed EEC limits. No chromium was detected in any of the samples analysed.

Preliminary tests with water cisterns made of glass fibre-reinforced plastics revealed no problems of metal extraction, but unidentified organic compounds were shown to be extracted during the first few months after installation. It was decided that the problem should be pursued with the British Standards Institution, with the object of minimizing the extraction of organic materials by control of tank manufacture. The Laboratory also made routine analyses of the acrylamide content of polyacrylamide resins, used as coagulant aids in the process of water clarification, on behalf of the Directorate General of Water Engineering of the Department of the Environment. Only three were found to have a monomer content exceeding the maximum specified concentration of 0·05% m/m, two being only marginally above this level.

In connexion with the EEC Directive on materials and articles intended to come into contact with foodstuffs, the Laboratory has been involved in a series of collaborative studies in which various methods of determining total migration have been evaluated. For predicting migration from plastics into fatty foods, a test method using liquid scintillation counting following contact with a ^{14}C-labelled synthetic triglyceride mixture was found to give the most reproducible results, but even then not with all plastics. Other methods were susceptible to errors resulting from incomplete extraction and to interference from additives already present in the plastics at the gas–liquid chromatography stage, and variations in total migration about a mean value could exceed 100% of that mean.

The development of field tests for toxic substances in industrial atmospheres continued to be one of the main functions of the Toxic Substances in Air Subdivision. The test originally developed for α-naphthylamine was shown to be applicable to other aromatic amines, and a new field test for mercury vapour was completed. Work was also started on the revisions of existing tests for benzene and isophorone, following proposed reductions in the TLVs for these compounds. Demands on the dust sampling and analysis service increased fivefold during the year, largely as a result of increased concern over the health hazards of asbestos, and surveys were conducted in many government department buildings to determine the contamination level and type of asbestos present.

Fd Cosmet. Toxicol. Vol. 16. pp. 283–285. Pergamon Press 1978. Printed in Great Britain

BOOK REVIEWS

Progress in Drug Metabolism. Vol. 2. Edited by J. W. Bridges and L. F. Chasseaud. John Wiley and Sons Limited, Chichester, Sussex, 1977. pp. ix + 348. £13.50.

This book is the second volume in a series that aims, by critical reviews, to keep the reader up-to-date on developments in various aspects of xenobiotic metabolism (*Cited in F.C.T.* 1977, **15**, 239). Six reviews are presented, each with its own list of references, together with both author and subject indexes covering the entire volume.

The first chapter, by G. J. Dutton and B. Burchell, deals with the important phase II conjugation reaction of glucuronidation. In addition to considering the mechanism of glucuronide formation and the properties of the enzyme(s) involved, the authors describe the factors that affect the glucuronidation process. Among these factors are age, sex, species, strain, diet and hormones.

Chapter 2, by J. R. Fry and J. W. Bridges, describes the use of isolated cell systems. After studies in the intact animal, the research worker is confronted with various *in vitro* techniques, including the use of isolated perfused organ systems, tissue slices, isolated cell suspensions, tissue homogenates and subcellular fractions. The authors suggest that whilst isolated perfused organ systems, such as the liver or lung, are time-consuming and suffer from certain technical problems, the use of 'microsomes' and other isolated subcellular fractions may, for a variety of reasons, lead to artefactual results. However, the use of isolated cell suspensions in studies of xenobiotic metabolism permits investigation of the entry of the drug into the cell, together with metabolism by both phase I (biotransformation) and phase II (conjugation) pathways. The authors describe the use of various types of freshly isolated cell suspensions (of hepatocytes for example) and cultured cells (including organ, explant and cell cultures) in metabolism studies, and pay particular attention to the metabolism of polycyclic hydrocarbons by various cell or tissue-culture systems.

The transplacental transfer of foreign compounds is one area of xenobiotic metabolism that has received much attention in recent years. In the third chapter of this book, O. Pelkonen reviews this subject and considers the metabolism of xenobiotics by foetal tissues and placenta derived from both human and animal sources. In the human foetus, the major sites of foreign compound metabolism are the liver and adrenal glands which, together with the placenta, possess cytochrome *P*-450-linked electron-transport systems capable of metabolizing a wide variety of xenobiotics and steroids. Attention is also devoted to the properties of cytochrome *P*-450 derived from both placental and foetal tissues, the induction of xenobiotic metabolism in these tissues and the significance of drug-metabolizing enzymes in the development of the foetus.

The next two chapters are devoted to methodology, dealing in turn with the use of stable isotopes and ion-pair extraction methods. Stable isotopes (such as ^{13}C, ^{2}H and ^{15}N) offer certain advantages over radioactive isotopes (^{14}C and ^{3}H), particularly in studies of the metabolism of drugs with a long half-life, since such studies could require longer exposures than would be deemed desirable for radioactive isotopes. The detection of stable isotopes is accomplished by the techniques of nuclear magnetic resonance and mass spectrometry. The author of this chapter, D. R. Hawkins, gives many examples of the use of stable isotopes in xenobiotic metabolism studies and his test is well illustrated with structural formulae, mass spectra and other figures.

The technique of ion-pair extraction involves the extraction of ionized compounds, including both free and conjugated drug metabolites, from an aqueous solution into an organic phase by the addition of an oppositely charged counter ion. This technique is applicable to both anionic and cationic organic compounds and may be advantageous in the isolation of labile compounds, as the extraction procedure can be performed under non-destructive conditions. A group from Uppsala, led by G. Schill, considers the theoretical aspects of this technique and illustrates, with many examples, its application in the extraction, separation and purification of drug metabolites.

The final chapter, by G. L. Mattok and his colleagues from the Drug Research Laboratories, Ottawa, deals with aspects of drug bioavailability and dissolution from various therapeutic formulations. The authors describe the concept of the relationship between a drug's absorption from a particular dosage form and its dissolution from the same dosage form. Hence by a simple *in vitro* physico-chemical test—the measurement of the dissolution of a drug from a soluble oral dosage form—an estimation may be made of the bioavailability of the drug to the patient. The theoretical aspects of both bioavailability and drug dissolution are considered and *in vivo* and *in vitro* data are reviewed for 23 drugs in a variety of formulations.

As with the first volume of the series, the Editors have assembled in one book a number of excellent reviews on various aspects of xenobiotic metabolism. One probable drawback of compilations such as this must be that only one or two reviews in any one volume may be of interest to a particular reader. Whilst this may reduce sales to private individuals, the overall quality of these reviews will ensure that each volume becomes recognized as a most useful work of reference. This volume is very highly recommended.

Foreign Compound Metabolism in Mammals. Vol. 4. Senior Reporter D. E. Hathway. The Chemical Society, London, 1977. pp. xiii + 411. £27.50.

The aim of this series is to collate the literature published in the field of mammalian xenobiotic metabolism. Volumes 1, 2 and 3 of this series, previously

reviewed (*Fd Cosmet. Toxicol.* 1972, **10**, 693; *ibid* 1973, **11**, 1116; *ibid* 1976, **14**, 52), covered the literature published up to 1973. The current volume reviews that published in the 2-year period 1974/1975 and consists of four chapters. It lists, in all, over 2000 references and provides compound and author indexes, but unfortunately, in common with its predecessors, it contains no subject index. Furthermore, the compound index is not cumulative in respect of the data presented in earlier volumes.

The first chapter, by P. G. Welling, deals with the pharmacokinetics of various classes of drugs and other xenobiotics. Chapter 2 consists of two parts, in the first of which D. E. Hathway reviews literature on the metabolism of certain drugs, pesticides, food additives, carcinogens and other environmental contaminants, while in the second C. T. Bedford considers specific agricultural and industrial chemicals. As with chapters 3 and 4, the text is well illustrated with the structures of many of the compounds under discussion.

In the third chapter, the various mechanisms of phase 1 (biotransformation) and phase II (conjugation) reactions involved in the metabolism of foreign compounds are reviewed by D. H. Hutson. Some attention is also given to the properties of mixed-function oxidase enzymes, including cytochrome *P*-450, and to the literature on both the induction and inhibition of xenobiotic metabolism. The fourth chapter, by J. D. Baty, deals with species, strain and sex differences in foreign-compound metabolism. Many examples of species differences in both biotransformation and conjugation reactions are given and some attention is paid also to quantitative differences in metabolism between different sexes, strains and species of animals.

Whilst the various authors have successfully reviewed a large number of publications, this volume can be recommended only as a work of reference and not for general reading. The lack of adequate indexing to allow rapid retrieval of the information presented must therefore be considered a major deficiency in this somewhat highly priced series.

Liver Cell Cancer. Edited by H. M. Cameron, D. A. Linsell and G. P. Warwick. Elsevier Scientific Publishing Company, Amsterdam, 1976. pp. xv + 292. Dfl. 109.00.

Both clinical and experimental material are included in this book, and although one might wish for a better balance, the authors have made some attempt to relate the two fields.

The chapters dealing with clinical aspects form the major part of the book and cover the geographical distribution of liver cancer, its pathology, the clinical problems associated with its diagnosis and the current situation with regard to its treatment. A whole chapter has been devoted to the role of α-foetoprotein in the diagnosis of liver-cell cancer and in monitoring its progress. It's all good stuff, but because of the clinical orientation there is little reference to the excellent work carried out in experimental animals by G. I. Abelev and others, whose studies might well throw some light on obscure phenomena, such as the elevation of serum α-foetoprotein in non-neoplastic conditions associated with liver-cell hyperplasia.

The chapter on the induction of liver-cell cancer by chemicals is largely 'old hat' and consists basically of an uncritical presentation of data relating to tumour induction by a whole variety of hepatocarcinogens. The author makes no distinction, in terms of human hazard, between carcinogens that, like aflatoxin, induce cancer when administered in very small doses and those that do so after prolonged administration of high doses. The chlorinated hydrocarbons are in the latter group and yet the author seems to be not a little surprised that, in contrast to the position with aflatoxin, there is no epidemiological evidence that the chlorinated hydrocarbons are capable of producing cancer in man, despite the fact that they have been used commercially for years for a variety of purposes.

Two chapters in this book deserve the close attention of the experimentalist interested in mechanisms of hepatocarcinogenesis. One, by V. M. Craddock, reviews the evidence that points to the particular susceptibility of replicating liver cells to the action of chemical carcinogens. The author brings together a variety of data from the field of biochemistry and molecular biology in an attempt to throw some light on the basis for this particular susceptibility of the dividing cell. Although, as she admits, no real explanation is available at the moment, the data are presented skilfully in such a way as to leave little reasonable doubt that "the initiation of the carcinogenic process may result from the replication of DNA which takes place before the damage has been repaired....".

The other chapter deserving particular attention is one by E. Farber dealing with the pathology of experimental liver-cell cancer. The morphological aspects of the mode of origin of the proliferative lesions induced by carcinogens are very well covered and the author expresses some views on their meaning in the induction of liver cancer. Unfortunately, he lays little stress on the importance of hepatocellular injury in the induction of cancer although he covers well the progression to fully developed cancer from the hyperplastic nodule, which is a reaction to hepatic injury.

This book has rich pickings for both the clinician and the experimentalist. The toxicologist, however, is likely to be disappointed if he expects to discover some better way of assessing the experimental induction of liver cancer in terms of human hazard than is available at the moment.

Enzyme Patterns in Fetal, Adult and Neoplastic Rat Tissues, By W. E. Knox. 2nd Ed. S. Karger, Basel, 1976. pp. xxiv + 359. Sw.fr. 49.00.

It has been said many a time that neoplasia is a reversion from the adult stage to the foetal. The adult stage is considered to be 'fully differentiated', the epitome, so to speak, of specialization in both the structure and function of the tissues. Reversion to the foetal stage is designated 'dedifferentiation', a term frequently used by morphologists to describe the degree of malignancy in cancer—the more dedifferention observed in a tumour, the greater the degree of malignancy.

Loss of differentiation is seen not only in morphology but also in the pattern of enzymes present in the tissues, a point competently brought out in the various chapters of the book named above. Emphasis is placed mainly on the enzyme characteristics of adult tissues in many species, including man, and on the patterns of enzymes in various tumours that arise in these tissues. Unfortunately, little attention is paid to foetal tissues, despite the prominence of this aspect in the title, but no doubt this is largely a reflection of the paucity of available information on the foetus and neonate. The data that are available, however, seem to suggest that neoplastic enzyme patterns bear a closer resemblance to the foetal than to the adult situation.

It is interesting to observe that the work devoted to enzyme patterns in normal and neoplastic tissues apparently bears out an observation made by J. P. Greenstein as long ago as 1947: "Tumours tend chemically to resemble each other more than they do normal tissues or than normal tissues resemble each other".

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Information Section

ARTICLES OF GENERAL INTEREST

HEXACHLOROBENZENE METABOLISM—MAINLY IN THE RAT

Hexachlorobenzene (HCB) is used as a fungicide, and is also a by-product of the industrial synthesis of many chlorinated hydrocarbons (*Cited in F.C.T.* 1976, **14,** 351). It has hepatotoxic potential (*ibid* 1977, **15,** 80) and studies of its metabolism are particularly important because of the suspicion that a metabolite may be responsible for the porphyrogenesis which is a feature of HCB intoxication (*ibid* 1976, **14,** 352).

Iatropoulos *et al.* (*Envir. Res.* 1975, **10,** 384), in the course of a study of the absorption, transport and distribution of dichlorobiphenyl, dieldrin and HCB, gave a single intragastric dose of 150 μg ^{14}C-labelled HCB to male and female rats, which were examined 1–48 hours later. Little HCB was absorbed by the gastric and duodenal walls during the first hour after dosing, but after 3 hours increasing concentrations appeared in the cells lining the jejunum and ileum. Some ^{14}C activity appeared in the liver and kidney, principally some 5 hours after dosing, but the highest levels were in the lymph nodes and adipose tissue and these levels were maintained throughout the observation period. Thus, absorption of HCB appears to be slow, with only minor involvement of the portal venous system; most absorption is effected by the lymphatic system and leads to deposition in body fat.

Deposition in body fat was also a marked feature of the tissue distribution of HCB fed to rats for up to 15 weeks in daily doses of 0·5–32 mg/kg (Kuiper-Goodman *et al.* (*Toxic appl. Pharmac.* 1977, **40,** 529). In the rats killed between weeks 3 and 15 of the feeding period, the tissue levels were relatively constant, those in adipose tissue, the liver, and the brain, kidney and spleen, respectively, being some 200–500, 10–20 and 5–10 times higher than the serum levels. Tissue concentrations of HCB declined slowly during a 33-week period in which some of these rats were given an HCB-free diet. No striking difference between the sexes was apparent in the tissue-level determinations after a single HCB dose (Iatropoulos *et al. loc. cit.*) but in the feeding study HCB concentrations in the adipose tissue and brain were considerably higher in females than in males, and serum levels were also generally higher in females. This paper (Kuiper-Goodman *et al. loc. cit.*) thus provides support for previous demonstrations of a sex-difference in the tissue accumulation of HCB in rats and in the greater sensitivity of females of this species to the toxic effects of this compound (*Cited in F.C.T.* 1976, **14,** 351).

Conflicting evidence on this aspect was presented, however, by Villeneuve & Newsome (*Bull. env. contam. & Toxicol.* (*U.S.*) 1975, **14,** 297), who gave pure HCB in daily oral doses of 500 mg/kg to groups of eleven rats and seven guinea-pigs of each sex for up to 16 days. On the basis of death rates and body-weight loss, the male rats appeared slightly more sensitive than the females, with eight males and five females dying during treatment. There were no significant differences between the sexes, however, in the brain and liver levels of HCB, either in the animals that died or in the survivors, which were killed 24 hours after the last HCB dose. It seems likely that the greater susceptibility of female rats compared with males is limited to the porphyrogenic properties of HCB. Porphyria only develops after about 3 weeks or more of HCB administration, depending on the dose, and would therefore not affect the results of acute or short-term studies like that of Villeneuve & Newsome (*loc. cit.*). These authors also reported that among the guinea-pigs, all of which died during treatment, the males lost more weight and accumulated more HCB in the brain and liver than the females. In comparison with the rat, tissue accumulation of HCB was lower in the guinea-pig, although the rat was less susceptible to the toxic effects of HCB in terms of deaths and body-weight loss.

When female rats were fed pure HCB at a dietary level of 80 ppm from 2 weeks before mating until the killing of their second litter, examination of the pups from each litter at the age of 18 days showed the highest concentrations of HCB in the liver, followed in diminishing order by the kidney, lung, brain, spleen and heart (Mendoza *et al. Envir. Physiol. Biochem.* 1975, **5,** 460). No sex-determined differences in concentration were apparent. Liver concentrations of HCB were about 60 ppm. The porphyrin concentration of the livers of exposed pups was about 2·5 times the control level, and was similar in males and females. The liver weight of offspring was raised significantly by HCB treatment of the dams, whereas the weights of kidney, brain, spleen and heart were reduced, the decrease in brain weight being greater in females than in males.

With the exception of the work of Iatropoulos *et al.* (*loc. cit.*), which involved carbon-14 determinations after administration of [^{14}C]HCB, all the studies mentioned above were based on the gas-chromatographic determination of unchanged HCB in the blood or tissues. Several subsequent papers have been concerned with the identification of possible HCB metabolites in the tissues and excreta of rats.

Engst *et al.* (*Bull. env. contam. & Toxicol.* (*U.S.*) 1976, **16,** 248) intubated male rats daily with 8 mg HCB/kg for 19 days, after which the liver, kidneys, adrenals, heart, spleen and intestinal fat were isolated and *n*-hexane extracts were subjected to gas chromatography. Urine and faeces were collected for analysis during the second and third weeks of HCB treatment. Pentachlorobenzene and pentachlorophenol were found in low concentrations in the organs and tissues examined. The mean concentrations of HCB in fatty tissue and muscle were 82 and 17 ppm, respectively, while the total amounts of HCB determined in the major organs averaged 125, 21, 9, 1·5 and 0·5 μg in

the liver, single kidney, spleen, heart, and single adrenal gland, respectively. Urine contained HCB and pentachlorophenol, which constituted the main metabolite, together with 2,3,4,6- and/or 2,3,5,6-tetrachlorophenol, 2,4,6-trichlorophenol, pentachlorobenzene and traces of 2,3,4- and other trichlorophenols. Small amounts of the glucuronides of 2,4,6-trichlorophenol and 2,3,4,6-tetrachlorophenol were also present. Faeces contained relatively large amounts of HCB and a little pentachlorobenzene. The main degradation route for HCB in rats thus appears to involve conversion to pentachlorophenol and subsequently to tetra- and then trichlorophenols. Another metabolite isolated from the urine of male and female rats given an oral dose of 300 mg HCB/kg was conclusively identified as 2,4,5-trichlorophenol by Renner & Schuster (*Toxic. appl. Pharmac.* 1977, **39,** 355), who thus confirmed and extended an earlier indication that traces of this compound were present in the urine of HCB-treated rats (Mehendale *et al. J. agric. Fd Chem.* 1975, **23,** 261).

In a more quantitative study of HCB metabolites, Koss *et al.* (*Arch. Tox.* 1976, **35,** 107) gave female rats ip injections of ^{14}C-labelled HCB on 2 or 3 occasions over a period of 5 or 10 days, to provide a total dose of 260 or 390 mg/kg. Urine and faeces were collected for 4 weeks after the initial injection, and tissues were isolated for analysis at the end of this period. The mean excretion of labelled carbon totalled 7% in the urine and 27% in the faeces. In the latter, about 30% of the activity was in the form of metabolites and 70% as unchanged HCB, while over 90% of the urinary activity was in the form of metabolites, principally pentachlorophenol, tetrachlorohydroquinone and pentachlorothiophenol. In the tissues, the only metabolite detected in measurable amounts was pentachlorophenol (accounting for 10% of the activity in blood, 3·5% in liver, 2% in kidney, 1% in brain and less than 0·1% in body fat), most of the remaining activity being accounted for by unchanged HCB. At the end of the experimental period (4 weeks after the first dose), about 65% of the administered dose remained, largely unchanged, in the body, about 18% had been excreted unchanged in the faeces and some 15% had been recovered as urinary and faecal metabolites.

We are left again with the problem of relating these findings to the human situation. HCB has been shown to be a virtually universal residue in human body fat and a study carried out among children in various parts of Upper Bavaria (Richter & Schmid, *ibid* 1976, **35,** 141) revealed concentrations of HCB in the blood ranging from 2·6 to 77·9 ppb ($b = 10^9$). Mean levels showed a rapid rise during the first 3 years of life but remained roughly constant for children between the ages of 5 and 18 years, at 22 ppb for boys and 17 ppb for girls. These levels are of roughly the same order as those found in earlier studies of human blood samples.

[P. Cooper—BIBRA]

A BREATH OF CADMIUM

Occupational exposure to dusts containing cadmium (Cd) can result in a deterioration of lung function, as shown by the standard tests for forced vital capacity, forced expiratory volume at 1 second, and peak expiratory flow rate (*Cited in F.C.T.* 1977, **15,** 479). Some further light has now been shed upon the mechanism by which Cd damages the lungs.

Palmer *et al.* (*Am. Rev. resp. Dis.* 1975, **112,** 173) exposed rats for 2 hours to an aerosol containing 0·1% cadmium chloride ($CdCl_2$) in physiological saline (generated from a 0·005 M solution and yielding an atmospheric concentration of 10 mg $CdCl_2/m^3$) and then killed batches of the animals for pulmonary investigation at intervals ranging from 1 hour to 10 days after treatment. The wet weight of the lungs of exposed animals (expressed as a percentage of terminal body weight) was doubled by day 4, but the only indication that this was due to inflammatory oedema was a transient increase in the fluid content of the lungs 24 hours after exposure. By day 10 the wet weight had dropped again almost to that found in untreated rats or in controls exposed to a saline aerosol, but by that time the dry weight of the Cd-exposed lungs showed a significant increase. In rats given an ip injection of tritiated thymidine 30 minutes before they were killed, there was a marked appearance of the ^3H label in type II alveolar cells 24 hours after inhalation of $CdCl_2$; this reached a peak at day 3, declining to the basal control level by day 7. This indication of appreciable cellular proliferation after Cd exposure was supported by a marked increase in the lung content of DNA, which reached a peak on day 4. From day 2 onwards, ^3H-labelled nuclei were present in the interstitial lung cells and in cells lying free in the alveoli as well as in the type II alveolar cells. Thus exposure to Cd aerosols evidently induced proliferation of lung cells, a development that may be related to fibrogenesis.

In another experiment using the same concentration of $CdCl_2$ aerosol (Hayes *et al. ibid* 1976, **113,** 121), the lungs from exposed rats were homogenized for the determination of total extractable lipid and of malate, lactate, isocitrate and glucose-6-phosphate dehydrogenases. By day 4 after exposure, at a time when wet weight and total DNA content of the lungs had approximately doubled, total lipid, lactate dehydrogenase and glucose 6-phosphate dehydrogenase had also doubled. A peak in malate dehydrogenase concentration 1 hour after Cd exposure was followed by an equally sharp fall and a subsequent slow rise over the next 4 days. Enzyme activities had returned to control levels by day 10, but the content of extractable lipid remained elevated. The changes found were generally consistent with a non-specific lung injury involving the proliferation of type II cells, but the sharp initial rise in malate-dehydrogenase activity

may have been an indication that there was also specific damage to the mitochondria, resulting in a leakage of mitochondrial enzymes.

Structural damage induced in the lungs of rats exposed for 2 hours to a 0·1% $CdCl_2$ aerosol has been described by Strauss *et al.* (*Am. J. Path.* 1976, **84,** 561). Multifocal damage centred on the bronchioles was seen by light microscopy. Ultrastructurally, type I-cell oedema with loss of surface plasma membranes was evident at 24 hours, and by day 2 there was marked proliferation of type II cells. By day 3, damaged alveoli were seen to be lined with plump cuboidal cells, which became progressively flatter after 4–7 days and recovered the appearance of type I cells by day 10. This pattern of replacement of injured type I cells by proliferating type II cells, which in turn gave rise to flatter cells without osmiophilic bodies and surface microvilli and thus to the regeneration of type I epithelium, closely resembled that induced by a number of toxic gases, including high concentrations of ozone, oxygen and nitrogen dioxide. There are indications of differences, however, notably that the increase in mitotic activity is apparently less prolonged in the latter cases than with Cd. Another difference—the multifocal rather than homogeneous nature of the Cd-induced response—may merely reflect the more restricted distribution of Cd droplets compared with the ready diffusion of an inhaled gas.

The possibility that Cd-induced lung damage may reduce resistance to inhaled infection has been explored by Gardner *et al.* (*Bull. europ. Physiopath. resp.* 1977, **13,** 157). Mice were exposed to $CdCl_2$ aerosols at atmospheric concentrations between 80 and 1600 μg Cd/m^3 for 2 hours, and were afterwards challenged with a viable aerosol of *Streptococcus pyogenes*. The number of deaths was 15–70% higher in animals exposed to $CdCl_2$ before bacterial challenge than in the control group and the mean survival times were reduced by 1–10 days, both parameters showing a linear relationship to the logarithm of the aerosol concentration. Deposition of Cd in the lungs of exposed animals ranged from 2 to 26 μg/g dry weight, according to the level of exposure. Even when animals were allowed 24 hours to recover in clean air before the bacterial challenge, mortality was increased by exposure to 550 or 1675 μg Cd/m^3. A delay in the clearance of streptococci from the lungs of animals exposed to $CdCl_2$ correlated with the observed increase in deaths.

Rats were used by Gardner *et al.* (*loc. cit.*) to determine the responses of various types of cells involved in respiratory defence systems to a 2-hour exposure to an aerosol concentration of 500 or 1500 μg Cd/m^3. There was a significant decrease in the total numbers of alveolar macrophages recoverable from the lungs by lavage immediately after the exposure, but normal values were recorded 24 hours later. The treatment had no significant effects on the numbers of lymphocytes recovered immediately after exposure, although there was some increase at 24 hours. The main effect, however, was on the total number of polymorphonuclear leucocytes recovered; after the higher exposure, these showed an immediate increase of 1·5 million (control value, 0·12 million) and an increase after 24 hours of some 13 million.

While these findings are clearly of interest in connexion with industrial exposure to Cd fumes, several of the authors mentioned above draw attention to their possible relevance to the question of lung damage associated with cigarette smoking. Cd tends to accumulate in smokers with chronic obstructive lung disease, particularly in those with emphysema (Hirst *et al. Am. Rev. resp. Dis.* 1973, **108,** 30; Lewis *et al. Lancet* 1969, **ii,** 1330; *idem, ibid* 1972, **i,** 291), and it appears to be derived directly from the cigarettes, each of which is estimated to have a Cd content between 1 and 4 μg.

[P. Cooper—BIBRA]

PCBs AND FECUNDITY

Polychlorinated biphenyls (PCBs) have long been recognized as serious environmental contaminants; they are extremely stable, widespread and likely to enter a number of food chains (*Cited in F.C.T.* 1975, **13,** 574 & 576). They have been shown to have deleterious effects upon reproduction in primates (Barsotti *et al. Fd. Cosmet. Toxicol.* 1976, **14,** 99) and their reproductive effects have recently been given closer scrutiny.

Sows fed 20 ppm PCB containing 42% chlorine (Aroclor 1242) throughout pregnancy and while nursing their offspring (Hansen *et al. Am. J. vet. Res.* 1975, **36,** 23) farrowed an average of 6·4 pigs per litter, compared with 11·4 per litter in controls. One sow that received PCB remained persistently sterile over 3 months. Weaning weights were similar in both groups but, excluding the sterile sow, those taking PCB weaned an average of 6·0 pigs compared with 8·4 in controls. Milk from treated sows contained less than 2 ppm PCB. Treated animals showed liver enlargement and their offspring had slight atrophy of spleen and thyroid. In two sows there were shallow focal erosions and congestion in the mucosa of the gastric fundus, but some controls showed similar though less severe lesions. No PCB residues were found in the spleen of three sows, but two had higher concentrations of PCB in spleen than in brain and ovary. The mean PCB content of fatty muscle was 2·5 ppm in sows and 1·8 ppm in offspring. Body fat contained 4–20 ppm PCB. Liver residues of PCB were not determined. Two sows treated with PCB developed chronic septicaemia, tentatively attributed to the immunosuppressive effect of PCB.

Male and female mice were fed 200 ppm PCB containing 54% chlorine (Aroclor 1254) for 60 days from the time of pairing (Merson & Kirkpatrick, *Bull. env. contam. & Toxicol.* (*U.S.*) 1976, **16,** 392). Nine litters were produced by 27 pairs fed PCB, whereas 33 litters

were produced by 26 control pairs. Only 29·6% of pairs fed PCB littered, compared with 84·6% of control pairs. Of the 27 pairs fed PCB, eight females and one male died between days 39 and 57 of the experiment, but none of the controls died. Litter size was not significantly affected by PCB feeding. It is suggested that PCBs may affect the maintenance of pregnancy by decreasing the level of progesterone and oestrogen in circulation because of an increase in steroid metabolism. This is supported by the study of Örberg & Kihlström (*Envir. Res.* 1973, **6**, 176) on the effect of PCB on the oestrous cycle of the mouse. Female mice were given 0·025 mg PCB (Clophen A60) daily for 62 days and then mated and examined on days 8–10 of pregnancy. The oestrous cycle was significantly prolonged by PCB treatment, and the frequency of implantation of ova was reduced. Such results may well arise from the effect of PCBs on the metabolism of sex hormones.

Prolongation of pregnancy by PCB treatment of mice has been reported by Török (*Bull. env. contam. & Toxicol.* (*U.S.*) 1976, **16**, 33). Mice were given 2,2′-dichlorobiphenyl in a daily oral dose of 375 or 750 mg/kg on days 1–3 of pregnancy, and were examined daily from day 17. Pregnancy in the treated animals was prolonged from a mean of 18·2 days in controls to 19·4 days in animals on the lower dose and 21·8 days in those on the higher one. Mean litter size was reduced from 11·5 to 7·9 and 4·8 respectively. In the high-dose group, a relatively low proportion (13%) of the dams carried litters. Once again the authors point to alterations in the balance of sex hormones as a possible explanation for delays in implantation and parturition.

In a study of the uterotropic activity of PCBs in rats, a series of six commercial PCBs (Aroclors) and pure mono-, di-, tetra- and hexachlorobiphenyls, with *o,p′*-DDT as a positive control, were injected ip in 8-mg doses into immature females (Ecobichon & MacKenzie, *Res. Commun. chem. Path. Pharmac.* 1974, **9**, 85). The animals were examined 18 hours later. Pronounced engorgement of the uterine blood vessels, with tissue swelling, followed administration of *o,p′*-DDT, but these effects were not seen consistently after the other treatments. Aroclor 1232 and 2,4,6,2′,4′,6′-hexachlorobiphenyl produced some dilatation of the uterine vasculature. Uterine weight and the levels of water and glycogen in the uteri were taken as indices of the uterotropic activity of the compounds. All three parameters were significantly increased by *o,p′*-DDT and by 2-chlorobiphenyl, while all the Aroclors except Aroclor 1016 significantly increased at least one of them. Compounds with a low chlorine content tended to increase uterine weight, while those with a higher chlorine content consistently increased uterine water and glycogen content. However, there was no correlation between type of uterotropic effect and degree of chlorination.

In studies on the pure isomers of chlorinated biphenyls, only 2-chlorobiphenyl affected tissue water and glycogen levels as well as uterine weight. The latter parameter was significantly higher following treatment with each of these compounds than in the dimethylsulphoxide-treated controls, but only the 2-chloro-, 2,2′-dichloro- and 2,4,6,2′,4′,6′-hexachlorobiphenyl treatments increased uterine weight significantly in comparison with biphenyl-treated rats. Despite its structural similarity to *o,p′*-DDT, 2,4′-dichlorobiphenyl was relatively inactive. Isomerically pure PCBs proved to be weak uterotropic agents, and it is possible that either the congener composition of the Aroclors or the presence of minor contaminants in them may contribute to such activity. Since the PCBs appear to be relatively resistant to biotransformation, metabolic products are unlikely to occur in sufficient concentrations to be responsible for any uterotropic activity, as has sometimes been suggested (Ecobichon & MacKenzie, *loc. cit.*).

Sanders *et al.* (*Toxic. appl. Pharmac.* 1977, **40**, 91) approached the problem differently, by studying the effects of PCBs on male mice. Two groups of 30 mice were fed a standard diet *ad lib.* or given 50% of the amount that the first group consumed *ad lib.* After 15 days each group was further subdivided and given either 0, 50 or 200 ppm PCB (Aroclor 1254) in the diet in the case of the *ad lib.* feeders or 0, 100 or 400 ppm PCB in the case of those receiving the 50% intake, so that the parallel subgroups in each main group received roughly equal weights of PCB. Administration of PCB in this way for 15 days led to a significant increase in liver weight and adrenal weight. Feed restriction also had a significant effect on adrenal weight but did not affect liver weight. Body weight was reduced by the diet restriction but not by PCB treatment. Pentobarbitone-induced sleeping times were significantly affected by both PCBs and food restriction, and the two factors interacted. Plasma corticoid concentrations were significantly increased by both PCBs and restricted diet and again there was interaction between the two factors. Unlike the restricted diet, PCB consumption did not have a significant effect on the weight of the reproductive organs of the mice, but it reduced significantly the sperm numbers per unit weight of testis. The latter effect could be the result of an alteration in hormonal balance, but it is not known whether reductions of the order of those observed in this experiment would appreciably affect fertility.

While there is no doubt that PCBs reduce fertility in the females of the species studied, significant effects on male fertility are less clear. Several of the authors cited see alteration of the hormonal balance, possibly by induction of the liver enzymes involved in steroid metabolism, as a likely mechanism for these effects.

[P. Cooper—BIBRA]

ALCOHOL AND THE FOETUS

The high levels of alcoholism recently publicized give the effects of foetal exposure to alcohol particular importance. It has been reported that, in rats, the consumption of ethanol before and during pregnancy

limits the number and size of progeny and increases malformations (*Cited in F.C.T.* 1976, **14,** 355), and there is also some experimental evidence that ethanol may act as a mutagen (*ibid* 1975, **13,** 581). Further information is now available from studies in rats, mice and man.

Studies in experimental animals

Female rats maintained on a diet containing 6% ethanol for an average of 21 weeks before pregnancy and for 20 days during it developed blood-alcohol concentrations of about 70 to over 150 mg/100 ml (Henderson & Schenker, *Res. Commun. chem. Path. Pharmac.* 1977, **16,** 15). Their offspring were examined at 3 days of age, after an injection of ^{14}C-labelled thymidine. The death rate among the newborn was considerably higher and the mean weight of the 3-day-old pups was significantly lower than in the controls. Protein concentration, indicated by ^{14}C uptake, was not significantly different from the control concentration in heart, liver and kidney, but was slightly raised in brain. All four organs showed unimpaired neonatal synthesis of DNA, but the liver had a significantly lower DNA concentration than was found in the control group. Total RNA concentrations in the four organs were depressed by 10–30%. Maternal exposure to ethanol therefore increases infant mortality, depresses growth and decreases the RNA concentration in vital organs of the rat.

The effect of ethanol exposure on neonatal hepatic-protein synthesis has also been investigated by Rawat (*Biochem. J.* 1976, **160,** 653), who treated pregnant rats with a single dose of 2 g ethanol/kg intragastrically or with a 6% ethanol diet, allowing newborns to be suckled for up to 10 days by mothers fed the 6% ethanol diet. Ribosome preparations derived from the livers of both adult and newborn animals exposed to ethanol showed inhibition of [^{14}C]leucine incorporation. Maternal exposure to ethanol resulted in a decrease in the total RNA content, RNA:DNA ratio and ribosomal-protein content of the foetal liver, without reducing its DNA content. Proteolytic activity and tryptophan-oxygenase activity were decreased in foetal, neonatal and adult livers after exposure to ethanol.

The *in vitro* incubation of normal adult rat-liver slices, but not of foetal liver slices, with 10 mM-ethanol inhibited the incorporation of [^{14}C]leucine (Rawat, *loc. cit.*). This ethanol effect on adult liver tissue was counteracted to some extent by the presence of pyrazole, which inhibits the action of alcohol dehydrogenase, but the addition of pyrazole to foetal liver slices incubated with ethanol made no difference to protein synthesis. These findings support the view that one factor in the adverse effect of ethanol on protein synthesis may be a change in the hepatic NADH/NAD$^+$ ratio, which is dependent on the activity of alcohol dehydrogenase. In view of suggestions that another factor in these responses to ethanol might be a change in corticosteroid balance, this study included the effect of the repeated alcohol intake on the activity not only of tryptophan oxygenase but also of tyrosine aminotransferase, since these two hepatic enzymes are known to be induced by corticosteroids. The results obtained, however, indicated that the effect of ethanol on these enzymes and on the rate of protein synthesis is not an indirect effect resulting from changes in corticosteroid levels, which have been shown by Ellis (*J. Pharmac. exp. Ther.* 1966, **153,** 121) to increase transiently in plasma after ethanol administration.

Kronick (*Am. J. Obstet. Gynec.* 1976, **124,** 676) has reported that ip injections of 7 g ethanol/kg (30 ml of a 25% (v/v) solution of 95% ethanol/kg) in pregnant mice were hypnotic, the animals remaining comatose for up to 4 hours. When this dose was given on days 8 and 9 or days 10 and 11 of pregnancy the incidence of foetal deaths was increased. In a more detailed study, the same dose of ethanol given in a single injection between days 9 and 12 increased foetal deaths, but when the dose was given on day 8 its effect on the death rate was only marginal. However, there was an increase in congenital abnormalities if ethanol was injected on day 8, 9 or 10, the commonest defects being coloboma of the iris, ectrodactyly of the forepaws, hypoplastic atria, hydronephrosis and exencephaly.

Pregnant mice given a liquid diet, in which 25% of the calories were in the form of ethanol, from day 5 to 10 of gestation (Randall *et al. Alcoholism clin. expl. Res.* 1977, **1,** 219) resorbed twice as many foetuses (16%) as did groups of controls either pair-fed a diet in which sucrose was substituted isocalorically for ethanol (6% resorbed) or fed *ad lib.* a standard laboraty diet (7%). However, since the number of implantations was also higher in the alcohol-fed group, the mean litter sizes were similar in the test and control groups, as were the foetal weights. More foetuses in the experimental group than in the controls showed malformations. Skeletal and heart anomalies were frequent and urogenital defects were particularly common.

Chernoff (*Teratology* 1975, **11,** 14A) has reported increased incidences of both foetal deaths and malformations in mice fed a liquid alcohol diet before and throughout pregnancy. Skeletal, cardiac and neural abnormalities were detected, and both the teratogenic and foetotoxic effects appeared to be related to the amount of alcohol consumed, in terms of the percentage of total calories derived from alcohol.

Human studies

Majewski *et al.* (*Münch. med. Wschr.* 1976, **118,** 1635) analysed 68 cases of embryopathy attributable to ethanol exposure. Of this group, 91% showed retarded intrauterine and postnatal growth, 87% microcephaly and 84% psychomotor and mental retardation, while 50% had genital abnormalities, 31% cardiac defects and 23% joint abnormalities. No correlation could be established between the quantity of alcohol consumed by mothers and birth weight, length of gestation or severity of symptoms, and it seems possible that defects in ethanol metabolism may account in part for the more severe effects. In a less extensive study, Ijaiya *et al.* (*Dt. med. Wschr.* 1976, **101,** 1563) observed similar signs in four children diagnosed as showing the foetal alcohol syndrome. They found a decrease in body length from birth, microcephaly, low intelligence and narrow palpebral fissures. Frequent congenital cardiac defects and some anomalies of the genitalia as well as other malformations were apparent. These defects were linked to severe alcohol abuse by the mothers.

In a study of the offspring of alcoholic women attending the outpatient clinic of a West New York hospital, Russell (*Alcoholism clin. expl Res.* 1977, **1**, 225) found that the average birth weight of babies born to such women was significantly lower than that of babies born to non-alcoholic women. Similarly, the incidence of babies that were premature and had very low birth weights was twice as high in alcoholic women as in the controls. There was an even greater excess (about three times the expected number) of babies with retarded intrauterine growth among those born to the alcoholic women. A number of socio-demographic factors were related to the lower birth weights. However, even when length of gestation, family income, number of children in the family and maternal education were taken into account, the onset of maternal drinking in relation to birth accounted for a 10% additional fluctuation in birth weight. No congenital malformations were recorded on the birth certificates of the 499 study children. This observation contrasts sharply with those of Majewski *et al.* (*loc. cit.*) and Ijaiya *et al.* (*loc. cit.*), but the apparent situation in the control group, and other factors, suggest that records were not adequate in this respect.

Ouellette *et al.* (*New Engl. J. Med.* 1977, **297**, 528) studied 322 offspring of women who were registered for prenatal care at Boston City Hospital. The women were categorized as abstinent or rare drinkers, drinking less than once a month (group 1), moderate drinkers, drinking more than once a month (group 2) or heavy drinkers, who occasionally drank five or more drinks and had a consistent daily average intake of more than 45 ml absolute alcohol (group 3). It was noted that heavy smoking was associated with heavy drinking. Of the offspring, 152, 128 and 42 had parents in groups 1, 2 and 3 respectively. The children were classed as 'abnormal' if they showed abnormalities either of a congenital nature, in growth or on neurological examination. Of the infants in group 3, 71% were 'abnormal' compared with 35% in group 1 and 36% in group 2. Hypotonia, jitteriness and inability to suck well afflicted a significantly greater number of offspring in group 3 than in groups 1 and 2. Prematurity, postmaturity and small size for gestational age were all markedly increased in infants of group-3 mothers, both length and weight at birth being significantly decreased. Congenital malformations were found in 32% of group 3, 9% of group 1 and 14% of group 2.

It has been pointed out in the *British Medical Journal* (1976, **II**, 1404), on the basis of evidence from numerous sources, that excessive ethanol consumption by expectant mothers is "a powerful cause of growth retardation and congenital malformations" in the infant. Maternal alcoholism seems to be responsible for a definite foetal syndrome involving maxillary hypoplasia, prominence of the forehead and lower jaw, short palpebral fissures, small eyes and epicanthic folds, and sometimes a squint or unilateral ptosis. Intrauterine growth is severely restricted and growth potential in childhood is poor, with almost universal microcephaly and mental retardation, accompanied by nervous inco-ordination and poor gross motor function. Other possible abnormalities are congenital heart disease, capillary haemangioma of the skin, ear deformities, hip dislocation, restriction of joint movement, abnormal palmar creases and sometimes hirsutism. Although other factors may be involved (heavy maternal smoking, poor maternal nutrition, poverty and infrequent clinic attendances being common associates of the heavy-drinking situation), ethanol is claimed to be the prime cause of this syndrome. It is easy to overlook, however, and the infant syndrome may often be attributed to adverse medico-social factors other than alcoholism.

Sneed (*J. Pediat.* 1977, **90**, 324) points out that some untaxed forms of alcohol (particularly in the south-eastern area of the USA) contain large amounts of lead and asserts that this factor does not seem to have been considered in articles on the foetal alcohol syndrome. This could be important since lead is a known teratogen. While this point may well deserve serious consideration, experimental studies using animals (cited above) have indicated the considerable teratogenic potential of ethanol alone. This suggests that although it may contribute, lead cannot account completely for the foetal alcohol syndrome.

In all but one of the human studies cited, a high alcohol consumption was associated with serious abnormalities in a large proportion of foetuses. The experimental studies in animals appear in general to substantiate these findings.

[P. Cooper—BIBRA]

Fd Cosmet. Toxicol. Vol. 16. pp. 293–304. Pergamon Press 1978. Printed in Great Britain

TOXICOLOGY: BIBRA ABSTRACTS AND COMMENTS

FLAVOURINGS, SOLVENTS AND SWEETENERS

3275. Aspartame metabolism in six species

Ranney, R. E., Oppermann, J. A., Muldoon, E. & McMahon, F. G. (1976). Comparative metabolism of aspartame in experimental animals and humans. *J. Toxicol. envir. Hlth* **2**, 441.

Aspartame (L-aspartyl-L-phenylalanine methyl ester) was found to be broken down in monkeys into methanol, aspartate and phenylalanine (PA), which were largely exhaled as carbon dioxide or (in the case of PA) incorporated into body protein (*Cited in F.C.T.* 1974, **12**, 778). Despite the liberation of free PA, aspartame appears to be well tolerated by phenylketonuric adolescents (*ibid* 1978, **16**, 77). These metabolic studies have now been extended by a comparison, in several different species including man, between the fate of aspartame labelled with ^{14}C in the methyl, aspartyl or PA moieties and that of the ^{14}C-labelled moieties in the free state. The materials were given by gavage to animals and were ingested by the human test subjects.

In rats there was no difference between the fates of the activity from [^{14}C-methyl]aspartame and from [^{14}C]methanol. In each case, peak levels of ^{14}C in the plasma were attained after 3 hr, and about 60% of the radioactivity appeared in the expired air within 8 hr. In the monkey, peak plasma levels of ^{14}C were reached later and $^{14}CO_2$ appeared more slowly after administration of [^{14}C-methyl]aspartame than after dosing with free methanol, but the total amounts expired over 8 hr (about 70% of the activity) were the same. The differences were ascribed, therefore, to the time taken by aspartame to reach the intestine and be hydrolysed by intestinal esterases, some of the methanol dose presumably being absorbed directly from the stomach.

Monkeys given [^{14}C-aspartate]aspartame also showed a slight delay in the appearance of $^{14}CO_2$ compared with those given free [^{14}C]aspartic acid. However, in both cases peak plasma levels of ^{14}C reached a maximum after 1 hr, and about 70% was expired in 12 hr.

When [^{14}C]PA or aspartame labelled in this moiety was given to dogs and monkeys, peak plasma levels of ^{14}C were attained after 5–7 hr, and a similar time-lag was observed in humans given [^{14}C-PA]-aspartame. In these species, and in mice, rats and rabbits, the proportion of radioactivity expired as CO_2 ranged from 7 to 24%, rabbits excreting the least and dogs the most by this route. In no case did more than a small proportion of the ^{14}C appear in the urine (0·4–5%) or faeces (0·3–6%). Analysis of plasma from rats, dogs, monkeys and man revealed no unchanged aspartame. The principal plasma constituents soon after [^{14}C-PA]aspartame administration were PA and tyrosine, and after 2–3 hr 90% of the ^{14}C had been incorporated into plasma protein.

These results support the theory that aspartame is hydrolysed by the intestinal esterases to yield methanol, which is oxidized to CO_2, and a dipeptide, which is split on the mucosal surface to free amino acids, which are absorbed. The aspartic acid moiety enters the tricarboxylic acid cycle and is converted largely to CO_2, whereas PA is principally incorporated into body protein, either unchanged or in the form of its major metabolite, tyrosine.

3276. The saccharin story: another instalment

Ball, L. M., Renwick, A. G. & Williams, R. T. (1977). The fate of [^{14}C]saccharin in man, and rabbit and of 2-sulphamoyl[^{14}C]benzoic acid in the rat. *Xenobiotica* **7**, 189.

Recent animal studies and epidemiological studies seem to be giving saccharin a stormy passage. The study reported below substantiates previous reports of its metabolic fate in animals.

Pregnant and non-pregnant Wistar albino rats and female Dutch rabbits were given a test dose of ^{14}C-labelled saccharin, after being fed either a normal diet, a saccharin-supplemented diet or, in the case of rabbits, saccharin-supplemented drinking-water, and the ^{14}C distribution and elimination were determined. Rats pre-fed with a saccharin-free diet eliminated 95% of the activity of the test dose in 24 hr, 76% in the urine and 14% in the faeces. No $^{14}CO_2$ was detected in expired air, no labelled carbonate was found in the urine and no metabolite appeared in either urine or faeces. A similar picture appeared in rats fed 1 or 5% saccharin in the diet for up to 12 months before the test dose. In bile-duct cannulated rats kept on a normal diet or an a 1% saccharin diet for 19 or 23 months before the experiment, 0·1–0·3% of injected labelled saccharin appeared in the bile within 3 hr and none after 24 hr. Most of the dose was excreted in the urine, with 0–6% in faeces. In pregnant rats given an oral dose of saccharin on day 21 of gestation, not more than 0·5% appeared in the foetus and this had decreased after 24 hr. The longest delay in clearance from maternal or foetal organs was from the urinary bladder. Liver microsomal preparations and foetal homogenates from rats kept on a normal diet or on a 1% saccharin diet for 2 years failed to metabolize saccharin *in vitro*.

Oral doses of [^{14}C]saccharin, given to rabbits that had taken a normal diet with or without 1% saccharin in drinking-water for 6 months, were excreted unchanged, 82–85% in urine and 5–11% in faeces 4 days after administration. In three adult volunteers, orally taken saccharin was excreted in 24 hr (85–92%) un-

changed, both before and after a daily dose of 1 g of saccharin for 21 days. Again no metabolite could be detected.

[^{14}C]Sulphamoylbenzoic acid, an impurity of saccharin, given orally to rats was excreted unchanged, but more slowly than was saccharin. No evidence of cyclization to saccharin *in vivo* was seen.

In accordance with previous studies (Byard *et al. Fd Cosmet. Toxicol.* 1974, **12,** 175), saccharin was rapidly excreted, mainly in the urine, and no metabolites were found. It is thought that the slight metabolism noted in some earlier studies may have resulted from impurities or from the methods used. The tendency for slow clearance of saccharin from the bladder is consistent with the recently publicized slight increases in bladder cancer amongst saccharin-fed rats (Arnold *et al. Science, N.Y.* 1977, **197,** 320).

3277. Cell metabolism of safrole, isosafrole and eugenol

Janiaud, P., Delaforge, M., Levi, P., Maume, B. F. et Padieu, P. (1976). Etude comparative en culture cellulaire de foie de rat du métabolisme de différents analogues et métabolites d'un hépatocancérogène naturel: le safrol. *C.r. Séanc. Soc. Biol.* **170,** 1035.

Investigations into the carcinogenicity of safrole (4-allyl-1,2-(methylenedioxy)benzene) have brought to light a large number of metabolites, several of which reacted electrophilically with nucleosides in a manner characteristic of known carcinogens (*Cited in F.C.T.* 1977, **15,** 645). Three of these metabolites were safrole epoxide (2′,3′-epoxysafrole), its hydroxylated product 2′,3′-dihydroxysafrole (1,2-methylenedioxy-4-(2,3-dihydroxypropyl)benzene) and 1′-hydroxysafrole, the last of which was shown to be far more carcinogenic than safrole in rats and mice (*ibid* 1974, **12,** 417). Analogous metabolites have now been demonstrated from isosafrole, which like safrole has induced hepatomas in mice (Innes *et al. J. natn. Cancer Inst.* 1969, **42,** 1101), and from eugenol (4-allyl-1-hydroxy-2-methoxybenzene), a flavouring which has apparently not been subjected to long-term testing.

When safrole was incubated in a culture of epithelial cells derived from adult rat livers, metabolites identified by gas chromatography–mass spectrometry were safrole epoxide, 2′,3′-dihydroxysafrole, 1′-hydroxysafrole, 3′-hydroxyisosafrole, 4-allylcatechol (1,2-dihydroxy-4-allylbenzene), 4-(2′,3′-epoxypropyl)-catechol, eugenol and 2′,3′-dihydroxyeugenol. Of these, all but the last had previously been reported as metabolites of safrole in the intact rat (*Cited in F.C.T.* 1977, **15,** 645). From isosafrole under the same conditions the chief product was 1′,2′-dihydroxyisosafrole, with lesser amounts of 1′,2′-epoxyisosafrole and 1′-hydroxysafrole, while eugenol gave rise principally to 2′,3′-dihydroxyeugenol. As in the intact rat, safrole epoxide was metabolized to 2′,3′-dihydroxysafrole. The epoxide hydratase inhibitor, 1,2-epoxy-3,3,3-trichloropropane (ETCP) greatly increased the yield of safrole epoxide from safrole and diminished that of allylcatechol, indicating that it was also blocking methylene transfer.

Although female rats are more resistant than are male rats to the hepatocarcinogenicity of safrole, female liver cells incubated with safrole gave rise to far more ethyl acetate-extractable epoxides and hydroxylated derivatives (4-allylcatechol, 2′,3′-dihydroxysafrole, 3′-hydroxyisosafrole and eugenol) than did male cells. However, unchanged safrole was also recoverable in greater quantities from female cells, and the findings were thought to have reflected differences in cellular fixation rather than metabolic oxidation. Support for this hypothesis was provided by studies with radiolabelled safrole, which showed that concurrent treatment with ETCP increased the proportion in the non-extractable phase to a far greater extent than that in the extractable phase. Other substances having the same effect as ETCP, although to a lesser degree, were epoxypropanol, isosafrole, safrole epoxide and hydroxysafrole, but eugenol, eugenol methyl ether and methylenedioxybenzene were without effect. Pre-treatment with 'cold' safrole decreased the amount of radioactive safrole in both phases, suggesting that the former was occupying some of the available binding sites.

After identical 24-hr incubations, safrole had killed 5% of female cells and 10–15% of male cells, its potency in this respect being greater than that of 2′,3′-dihydrosafrole but less than that of eugenol methyl ether, 2′,3′-dihydroxysafrole or safrole epoxide. The cytotoxicity of eugenol and of 2′,3′-dihydrosafrole was decreased by ETCP, suggesting that this property was not related to epoxide formation. Rather it appeared to depend on solubility and volatility, as well as on the opening of the methylenedioxy ring, and probably bore no relation to carcinogenicity.

EMULSIFIERS AND STABILIZERS

3278. Iota carrageenan the villain

Engster, M. & Abraham, R. (1976). Cecal response to different molecular weights and types of carrageenan in the guinea-pig. *Toxic. appl. Pharmac.* **38,** 265.

The three main forms of carrageenan differ slightly in the chemical linkages of their sulphated polygalactose units and in the number of sulphate groups present. Carrageenan obtained from *Chondrus crispus* consists of a mixture of kappa and lambda chains, whereas the material derived from *Eucheuma spinosum* consists predominantly of iota chains. Although the average molecular weight of the *C. crispus* carrageenan is very much higher than that of carrageenan from *E. spinosum*, both materials encompass a wide range of molecular weights. It has been indicated that molecular weight, configuration and route of administration are all factors in carrageenan toxicity (Pittman

et al. Fd Cosmet. Toxicol. 1976, **14**, 85). The polymer obtained from *E. spinosum* has been shown to have the greater ulcerative potential in sensitive animal species, and the study cited above attempted to identify the contributions of both structure and molecular weight to this toxic action.

Guinea-pigs were given 1% solutions of various fractions of carrageenan (identified throughout this note by their number average molecular weight) as their sole source of drinking-water for 2 wk. Three fractions of kappa (8500–314,000), three fractions of lambda (20,800–275,000) and seven fractions of iota (5000–145,000) carrageenan were used. Faecal blood was observed on occasions in the animals receiving the 8500 kappa fraction or the 74,800 lambda fraction. Small haemorrhagic lesions occurred in the caeca of five animals receiving the iota fractions 39,000, 88,000 or 107,000 and in one control animal.

There were no histological abnormalities in the animals receiving kappa or lambda carrageenan of any molecular weight or in those ingesting iota polymers of the highest and two lowest molecular weights (145,000, 8700 and 5000). Thinning of the absorptive epithelium, together with large foamy macrophages in the lamina propria, crypt abscesses and cellular infiltration, occurred in the caeca of all the guinea-pigs receiving the other iota fractions. The ulceration produced by the 21,000 and 39,000 fractions was accompanied by an inflammatory response. Acid-phosphatase activity, which was very slight in the controls, was apparent in the animals given iota carrageenan and was particularly intense in the macrophages under the observed areas of epithelial thinning. Migration of lymphocytes and macrophages into the epithelium, a finding not reported previously, occurred in animals given iota fractions 8700, 21,000 and 39,000. Many of these macrophages and those in the submucosa contained carrageenan-like material. An increased number of macrophages was seen in the caecal lamina propria of all iota-treated animals except those receiving the material of highest and lowest molecular weight.

In contrast to their activity when given in the drinking-water, the same iota-carrageenan fractions did not produce caecal ulceration when given at 2% in the diet of guinea-pigs for 10 wk. Body-weight gain was depressed, however, in animals receiving dietary supplements of iota fractions 8700, 21,000 and 39,000.

This study produced no evidence that kappa and lambda carrageenans of even the lowest molecular weights produced ulceration. While the iota polymer did show this toxic action, the material of highest and lowest molecular weight was not absorbed and stored in the macrophages and did not elicit ulcerative effects. Moreover, these experiments indicated that no lambda and very little kappa carrageenan was absorbed by the gut. The latter finding conflicts with earlier evidence (Pittman *et al. loc. cit.*) that all three types of carrageenan were absorbed in the guinea-pig's gastro-intestinal tract, and seem to support the contention that kappa and lambda carrageenans, which may be present in food, pose less hazard than does the degraded iota-chain material obtained from *E. spinosum*.

PRESERVATIVES

3279. Nitrosamine threat still viable

Diaz Gomez, M. I., Swann, P. F. & Magee, P. N. (1977). The absorption and metabolism in rats of small oral doses of dimethylnitrosamine. Implication for the possible hazard of dimethylnitrosamine in human food. *Biochem. J.* **164**, 497.

Nitrosamines have been shown to be carcinogenic in many animal species (Schmähl, *Fd Cosmet. Toxicol.* 1977, **15**, 475), and the discovery of their widespread occurrence in foods, although at extremely low levels, has inevitably generated concern about their carcinogenicity in man. *N*-Nitrosodimethylamine (NDMA) is known to be converted to an active alkylating agent not only in various rat tissues but also in human liver tissue (*Cited in F.C.T.* 1971, **9**, 296) and the reaction of this metabolite with tissue DNA has been postulated as the preliminary stage in the induction of tumours by NDMA. The study cited above provides indirect evidence to suggest that the levels of NDMA that are found in some types of food could present a significant carcinogenic hazard for man, although several questions remain open.

NDMA was administered by gavage to female Wistar rats at levels from 1 μg to 10 mg/kg body weight. The animals were killed 15 min, 30 min, 1 hr or 2 hr after dosing and the alkylation of liver and kidney DNA was determined by measurement of 7-methylguanine levels. The amount of methylation of liver DNA was directly proportional to dose throughout the range used, indicating that the fraction of loss was similar when both large and small amounts of NDMA were absorbed from the gut. Kidney methylation was detectable only at doses greater than 40 μg/kg body weight, but at these higher treatment levels alkylation was again dose-related.

At the highest doses with which, it was suggested, all the tissues would be equally exposed to NDMA, the amount of alkylation of the liver DNA was about four times that in the kidney, an indication of the relative NDMA-metabolizing power of the two organs. A similar ratio of alkylation occurred when NDMA was given iv. However, the liver was able to metabolize almost all the NDMA absorbed from the gut at the lower treatment levels, as shown by the increasing ratio of liver to kidney DNA alkylation with decreasing dose. At 40 μg NDMA/kg, the alkylation in the liver was about 75 times that in the kidney. Therefore the authors considered that the liver of the rat was able to afford, to the other organs, some protection from the effects of NDMA, although at some increased risk of hepatocarcinoma. Previous animal

studies support this view; large doses of NDMA produce kidney tumours in the rat whereas prolonged feeding of small doses leads to the formation of liver tumours but not kidney tumours (*Cited in F.C.T.* 1964, **2,** 61; Magee & Barnes, *Adv. Cancer Res.* 1967, **10,** 163.

This study indicates that the levels of NDMA found in certain constituents of the human diet are carcinogenic to the rat. The susceptibility of the human liver to NDMA carcinogenesis *in vivo* is unknown however, although *in vitro* studies suggest that the sensitivity of DNA to alkylation by NDMA is similar in human and rat liver (*Cited in F.C.T.* 1971, **9,** 296). In any attempt to extrapolate results of rat studies to possible human situations, it is also important to bear in mind that the level of absorption from the gut may vary widely in the two species. The destruction of NDMA by the gut bacteria has been demonstrated (Rowland & Grasso, *Appl. Microbiol.* 1975, **29,** 7) but the question of whether the human intestine itself can metabolize small amounts of nitrosamines to inactive derivatives has not yet been resolved.

PROCESSING AIDS

3280. A glut of phosphates

Raines Bell, R., Draper, H. H., Tzeng, D. Y. M., Shin, H. K. & Schmidt, G. R. (1977). Physiological responses of human adults to foods containing phosphate additives. *J. Nutr.* **107,** 42.

Phosphates are widely used in the processing of meat, poultry, cheese, soft drinks and other food items. It has already been hinted that excessively high phosphorus (P) levels in the diet might adversely affect calcium (Ca) metabolism, at least in some individuals (Joint ARC/MRC Committee on Food and Nutrition Research, *Food and Nutrition Research*, p. 162; HMSO, London, 1974), and further observations of such an effect are reported below.

Eight subjects, five men and three women, in good health and with normal eating habits, were given a control diet, free of phosphate additives and containing about 95 g protein, 700 mg Ca and 1 g P, daily for 4 wk. For a second 4-wk period they were given a similar diet that contained added phosphates and provided 700 mg Ca and 2·1 g P daily. The phosphated items included processed cheese, treated meats and potato chips, refrigerator quick rolls and turnovers, and carbonated beverages containing phosphoric acid in place of citric acid. All subjects reported soft stools or mild diarrhoea, and abdominal discomfort, when starting the P-enriched diet. These disturbances abated after 1 wk in all but two subjects who had mild intestinal upsets throughout the 4 wk. During the experimental period serum-P concentration and urinary excretion of P rose, while serum-Ca concentration and urinary excretion of Ca fell. The increase in urinary P excretion ranged from 25 to 222%, with a mean of 137%, and was significant in six subjects. The mean decrease in urinary Ca was 37% and this difference was significant in seven subjects. Six showed an increase in cyclic-AMP excretion during the high-P period, and all showed an increase in hydroxyproline excretion, from a mean of 27·92 mg/24 hr during the control period to 33·38 mg/24 hr during the high-P period. Depression of serum Ca became progressively more marked from 1 to 3 hr after a high-P meal, although the difference from control values was significant only at 1 and 3 hr; serum P increased within 2 hr and remained elevated at 3 hr. These effects resemble those of enhanced parathyroid activity in animals, and raise the question whether loss of bone mass during human ageing may be increased by an excess of dietary phosphates.

PROCESSING AND PACKAGING CONTAMINANTS

3281. Cadmium exposure from medical plastics

Knudsen, F. U. & Persson, P. (1977). Exposure to cadmium from medical plastic utensils: An examination by random sampling. *Dan. med. Bull.* **24,** 45.

Following extensive and increasing industrial use, cadmium is now considered to be a major environmental contaminant. Its toxic effects on the testes and kidneys are well known and associations with hypertension (*Cited in F.C.T.* 1972, **10,** 253) and with chromosome abnormalities in the leucocytes of human peripheral blood (*ibid* 1977, **15,** 158) have also been documented. The study cited above resulted from the finding of a much elevated cadmium level in urine collected from a child (38·8 μg/litre compared with a mean for 'reference urine' of 0·3 μg/litre). This level was traced not to poisoning but to the leaching of cadmium from the plastics material used in a common type of pre-packed urine-sampling set. An examination was made, therefore, of the cadmium content of the sodium chloride solution from seven urine-sampling sets of the same type and of the leaching of cadmium from the various parts of the sampling sets and from 12 different randomly chosen plastics catheters into 0·9% sodium chloride.

The cadmium content was determined by atomic absorption spectrophotometry, in conjunction with atomization in a heated graphite atomizer. Cadmium was found in all the samples of saline from the sampling-set bags, in concentrations ranging from 330 to 580 μg/litre (the drinking-water at the laboratory contained 1 μg/litre). Further tests confirmed that the plastics bag was the source of this cadmium, and

saline from a new type of bag subsequently used in these sets was found to be almost free of cadmium. Very little cadmium was extracted from other components of the sampling sets. On the other hand, considerable amounts (up to 200 μg/litre extraction fluid) were extractable from the coloured stubs of one third of the catheters, when these were heated for 30 min at 70°C, each in 10 ml of normal saline, although with all 12 catheters, only traces (0·1–2·5 μg/litre) were demonstrated in similar extractions of uncoloured parts intended for internal use.

Although any accumulation of cadmium as a result of even long-term or repeated use of such catheters must be assumed to be very small. the high cadmium levels demonstrated emphasize the increasing exposure of the population to cadmium in the air, foods, cigarettes and utensils.

[In Denmark, from which this paper originated, a new Act relating to drugs was passed in 1976 and made possible a more effective supervision of new medical devices. Prior to that, the quality of utensils for hospital use was supervised by the 'Utensil Commission' of the National Health Service. The supervision was arranged on a voluntary basis and consequently only comprised registered products. There now exists in the UK a draft British Standard on the evaluation of medical devices for biological hazards (BSI Document 77/60856, 25 February 1977). The document will apply to devices that are in direct contact with body tissues or fluids or with fluids that are to be introduced into the body and that have a mechanical or physical rather than chemotherapeutic function. Hence a more adequate supervision of these devices should ultimately be possible.]

THE CHEMICAL ENVIRONMENT

3282. Selenium as an anticarcinogen

Jacobs, M. M., Jansson, B. & Griffin, A. C. (1977). Inhibitory effects of selenium on 1,2-dimethylhydrazine and methylazoxymethanol acetate induction of colon tumors. *Cancer Lett.* **2,** 133.

The toxicology of selenium (Se) presents us with a paradox. Its role, as carcinogen, anticarcinogen or both, has not been clearly established (*Cited in F.C.T.* 1972, **10,** 869). Further evidence now indicates that Se plays a role in inhibiting the induction of colonic tumours by certain chemicals.

In groups of 14 or 15 rats given a weekly injection of 20 mg 1,2-dimethylhydrazine (DMH) or methylazoxymethanol acetate (MAM)/kg body weight for 18 wk, the incidence of colonic tumours was reduced by the addition of 4 ppm Se as sodium selenite to the drinking-water. With DMH as the carcinogen, the number of rats developing such tumours was reduced from 13 to 6, and the total number of tumours observed dropped from 39 to 11. Selenium had no significant effect on the number of rats that developed colonic tumours after MAM administration, but reduced the total number of tumours from 73 to 42. The transverse colon was the principal site of tumours (adenocarcinomas) induced by both carcinogens. The gross appearance of the colons, disregarding tumours, was more normal in those animals given Se in their drinking-water than in those treated with DMH or MAM alone. The Se treatment did not significantly affect weight gain in these rats.

3283. UV ink teething troubles

Emmett, E. A. & Kominsky, J. R. (1977). Allergic contact dermatitis from ultraviolet cured inks. Allergic contact sensitization to acrylates. *J. occup. Med.* **19,** 113.

Emmett, E. A., Taphorn, B. R. & Kominsky, J. R. (1977). Phototoxicity occurring during the manufacture of ultraviolet-cured ink. *Archs Derm.* **113,** 770.

Ultraviolet-curable inks (UV inks) have proved particularly suitable for the printing of heavy substrates and have made significant inroads into the paperboard packaging field. UV inks consist of conventional pigments dispersed in a polymeric vehicle containing, typically, polyfunctional acrylates, UV-reactive unsaturated polymers or oligomers and one or more photo-initiators. The toxicity of the materials used in the formulation of these inks is largely undefined, and more data are essential for any valid assessment of safety-in-use. Because of the known potential of acrylates to cause skin complaints (*Cited in F.C.T.* 1972, **10,** 569), some concern has been expressed over the dermal hazards associated with the handling of the uncured ink. Sensitization is difficult to reproduce in animal models and to a large extent identification of active materials is only possible in the course of clinical studies.

The first paper cited above concerns a workforce which experienced sensitization problems in the first 5 months of the manufacture of a UV ink. Eight of the 58 men involved developed an allergic contact dermatitis characterized by pruritic and erythematous eruptions, usually accompanied by papules, vesicules and scaling, in the exposed areas of the body. In those who consistently wore gloves, the hands were not involved in the dermatitis but the face and neck were affected, indicating that the reaction could have been caused by volatile fumes as well as by direct skin contact.

In patch tests, seven of the eight workers, none of whom had any history of atopy, reacted to 1% trimethylolpropane triacrylate (TMPTA), six responded to 1% hexane-1,6-diol diacrylate (HDDA) and four to 0·2% pentaerythritol triacrylate. Some of the observed responses may have represented cross-reactions, but as each worker could have been exposed to all of the materials this could not be evaluated. Whilst all the acrylates were patch-tested at a concentration shown to be non-irritant to healthy volunteers, the skin response of two workers to TMPTA and to HDDA was very severe, suggesting that the 1% concentrations used may have been too high in these specific cases.

Although the inks were reformulated to exclude the three allergenic acrylates, dermatitis persisted in two workers. Patch tests showed that this was due to their contact with an epoxy acrylate used in the ink. Another employee, who was not sensitized towards the other polyfunctional acrylate monomers, also reacted positively to this material and to two out of four commercial epoxy acrylates of differing chain lengths. Nevertheless he, in common with all other employees, was not sensitized to an epoxidized linseed oil acrylate also present in the ink.

Photosensitivity, which was observed in the same workforce, is the subject of the second paper cited above. After working with the inks, four subjects experienced a burning or smarting sensation when their bare skin was exposed to the sun. The sensation persisted throughout the period of exposure and the accompanying erythema and swelling lasted for several days. Although two of the men showed evidence of allergic contact sensitization to the polyfunctional acrylates, the clinical signs observed suggested additional photosensitivity.

Five photo-initiators used in the UV inks, namely benzophenone, thioxanthone, diethoxyacetophenone, Michler's ketone and technical amyl *p*-dimethylaminobenzoate (ADMAB), which was contaminated with some *o*-isomer, absorbed UV radiation above 250 nm and were therefore considered to be potential causes of the observed photosensitivity. Of these, only Michler's ketone, thioxanthone and ADMAB were phototoxic to Ehrlich ascites cells *in vitro*. Photopatch testing of the ink components was then undertaken in three of the employees exhibiting photosensitivity and in four employees free of any clinical symptoms but also working with the UV inks. Each component was applied to the skin on duplicate patches. After 24 hr one patch was removed, and the skin was washed with alcohol and exposed to the midday sun for 25 min or to the afternoon sun for 35 min. The other patch was removed after a further 24 hr and the reactions at both sites were read. On exposure to sunlight, undiluted ADMAB produced a localized burning sensation and an immediate weak skin reaction in six of the seven workers tested. The skin reactions were in most cases still evident 24 hr after the exposure. There were no reactions at ADMAB sites occluded from the sun or at sites treated with any of the other UV-ink components with or without exposure to sunlight. Similarly, no skin reactions were observed in any of the seven workers at sites photopatch-tested with 5% ADMAB in petrolatum. Two of the subjects who reacted to undiluted ADMAB in the presence of sunlight were examined further and were shown to be unresponsive to sunlight alone. Neither individual was sensitive to any of a series of established allergens or had any demonstrable circulating antinuclear antibodies.

These findings suggested that the reported photosensitivity would be more accurately described as a clinical phototoxic reaction to the undiluted absorber. The phototoxic character of ADMAB was confirmed in four individuals who had not been occupationally exposed to this initiator. Three of the subjects noted a localized burning sensation when the ADMAB-treated site was exposed to sunlight, and this was accompanied by erythema and in one case by prominent oedema. This skin reaction was diphasic; the original erythema and swelling faded soon after the end of the exposure period, but erythema and resulting irritation returned 4–5 hr later and lasted for 2–5 days. Sunscreening agents, notably 10% sulizobenzone, applied to the skin prior to the sun exposure, were shown to give protection from the phototoxicity of ADMAB.

[As the second study described here demonstrates that the responses elicited by amyl dimethylaminobenzoate were due to its phototoxic rather than photoallergenic properties, a modest improvement in factory hygiene should prevent a recurrence of the problem. The observed incidence of sensitization to the acrylates is a more difficult hurdle to overcome. Although the number of workers afflicted with the dermatitis would probably be reduced if contact with the allergen were minimized, a potent sensitizer may still exhibit some activity at very low exposures. Even if the ink manufacturer were able to improve his own plant conditions enough to lower the incidence of sensitization to an acceptable level, his customers, possibly a multitude of small printers occupying less than ideal premises, might find suitable improvements in working conditions difficult to make. Clearly the safest course, and the most satisfactory if it is a practical possibility, is a policy of reformulation, with removal of the offending compounds, and this was the policy adopted by the manufacturer involved in the cases discussed here.

On a more academic level, the individual who was shown to be sensitized to epoxy acrylates of only certain chain lengths would seem to be unusual. No details are given of the four epoxy formulations tested, and so the observed differences in sensitizing ability may only have been a reflection of differing acrylate monomer levels. However, if the epoxy acrylate formulations were comparable in this respect, the relationship between allergenicity and chain length warrants further study.]

3284. Hazards of fluorinated contaminants

Bedford, C. T., Blair, D. & Stevenson, D. E. (1977). Toxic fluorinated compounds as by-products of certain BF_3-catalysed industrial processes. *Nature, Lond.* **267**, 335.

The hazards of inorganic fluorine compounds have been well documented (*Cited in F.C.T.* 1971, **9**, 872), but with the increasing use of fluorinated materials, particularly in the industrial sector, additional information on the toxicity or occurrence of both organic and inorganic fluorine compounds is clearly of value.

The preliminary note cited above is intended to draw attention to the possible presence of low levels of fluorinated compounds, some of considerable toxicological importance, in compounds prepared with boron trifluoride catalysts, and particularly in some products manufactured from ethylene oxide. Toxicity studies on some products in the latter category suggested that they might be contaminated with fluoracetate precursors, and chemical analyses confirmed the presence of low concentrations of 2-fluoroethanol and/or 2-(2-fluoroethoxy)ethanol, two compounds

that are very toxic to mice, with LD_{50} values of only 10 and 15 mg/kg, respectively. The former compound is the formal addition product of ethylene oxide and fluoride ion and gives rise to 2-(2-fluoroethoxy)-ethanol by further ethylene oxide addition. The possible involvement of free fluoride ion—perhaps liberated by solvolysis of the catalyst or derived from silicon tetrafluoride, a known contaminant of commercial boron trifluoride—in the formation of toxic by-products of these manufacturing processes has been supported by the finding that tetrafluoroborate ion, readily formed by interaction of boron trifluoride and free fluoride ion, was present as a contaminant of some of the products examined.

3285. Beware benzene!

Infante, P. F., Rinsky, R. A., Wagoner, J. K. & Young, R. J. (1977). Leukaemia in benzene workers. *Lancet* **II**, 76.

Benzene has an unenviable reputation for toxicity on inhalation and percutaneous absorption, and many cases of leukaemia have been reported in individuals who have worked in industries where outbreaks of acute or chronic benzene poisoning have occurred (*Cited in F.C.T.* 1977, **15**, 652). We now add one more piece of evidence to benzene's unhappy tale.

A comparison was made between workers occupationally exposed to benzene, in the manufacture of a natural-rubber film at two plants in Ohio during 1940–49, and two control populations, as part of a follow-up operation in 1975. Some 25% of the 748 men in the study group were assumed to be alive in the absence of any definite information, although the inaccuracy of this assumption was apparent from the discrepancy between the 140 known deaths in the study groups and the expected figure of 187·6. Even so, the study group showed a significant excess of actual over expected deaths from malignancy of the lymphatic and haemopoietic systems, attributable almost entirely to an excess of deaths from myelogenous or monocytic leukaemia. Seven leukaemia deaths in the study group contrasted with an expected 1·38 based on the rate for white US males (first control group) or 1·48 based on the rate for fibre-glass workers (second control group). The period elapsing from initial benzene exposure to death from leukaemia was 2–21 yr.

On the basis of data on the proportion of myelomonocytic leukaemias among the total cases of leukaemia occurring in the US white male population during a later period, the number of such cancers to be expected among benzene workers was 0·6967 compared with the seven observed, suggesting that exposure to benzene carries roughly a ten-fold increase in the risk of death from myelogenous and monocytic leukaemia.

It appears that the working atmosphere in the two plants involved in this study was not contaminated with any other solvent, and the levels of benzene were generally below the limits recommended at the time of their measurement. These recommended limits have gradually been reduced in the USA from 100 ppm (MAC) in 1941 to 10 ppm (8-hr time-weighted average) since 1969.

3286. A whiff of kerosene

Wolfsdorf, J. (1976). Kerosene intoxication: An experimental approach to the etiology of the CNS manifestations in primates. *J. Pediat.* **88**, 1037.

Wolfsdorf, J. (1976). Experimental kerosene pneumonitis in primates: Relevance to the therapeutic management of childhood poisoning. *Clin. expl Pharmac. Physiol.* **3**, 539.

The pneumonitis that results in children from ingestion of kerosene, an illuminating fuel consisting mainly of saturated and unsaturated aliphatic hydrocarbons, has been attributed to direct aspiration of the fuel rather than to absorption and excretion into the lungs (*Cited in F.C.T.* 1971, **9**, 752). Evidence now available suggests that the central nervous disturbances sometimes associated with kerosene poisoning are mainly due to the irritation and consequent hypoxia caused by such aspiration.

Baboons in which pneumonitis was induced by injection of 0·3 ml kerosene/kg intratracheally were killed 4 days later, and others given an injection of the same dose into the heart (by catheter to the left ventricle via the femoral artery), into the carotid artery or into the portal vein were killed 2 hr after treatment. No macroscopic or microscopic morphological abnormality was evident in the brains of any of the animals. Slight oedema was present in the brains of baboons given the intracardiac or intracarotid injection and to an even less marked degree in the group treated intratracheally. Transient convulsions occurred after the intratracheal dose but not after any of the others. In animals given an intratracheal or intracarotid dose, the lungs appeared oedematous and haemorrhagic. In those receiving the intraportal dose, some patches of cloudy swelling and haemorrhage appeared in the liver, but there were no differences between this group and the controls in the dry weight/wet weight ratios.

These findings indicate that the primate brain is highly resistant to the direct toxic effects of kerosene, even when doses injected directly into the circulation result in blood levels far in excess of those encountered clinically. The liver removes sufficient circulating kerosene to protect the brain against any toxicity resulting from systemic absorption, and the most potent cause of the central nervous disturbances that follow kerosene ingestion by children appears to be the hypoxia that results from pneumonitis after aspiration.

Further work by the same author was designed to investigate the common assumption that secondary bacterial contamination is an inevitable result of the acute chemical pneumonia and abscess formation associated with kerosene aspiration. Intratracheal injection of 0·3 ml kerosene/kg resulted in haemorrhagic pneumonitis, pleural effusions, fibrinous mediastinitis and abscess formation in eight of the ten baboons treated, and subsequently (by day 4) most of these showed evidence of acute fibrinopurulent pneumonia, bronchitis, pleurisy and micro-abscess formation. No aerobic or anaerobic bacterial contamination was detected, however, in any of the animals either by investigation of lung biopsies or, on day 4, by post-mortem studies.

3287. Styrene oxide in mutagenicity studies

Loprieno, N., Abbondandolo, A., Barale, R., Baroncelli, S., Bonatti, S., Bronzetti, G., Cammellini, A., Corsi, C., Corti, G., Frezza, D., Leporini, C., Mazzaccaro, A., Nieri, R., Rosellini, D. & Rossi, A. M. (1976). Mutagenicity of industrial compounds: Styrene and its possible metabolite styrene oxide. *Mutation Res.* **40,** 317.

Parkki, M. G., Marniemi, J. & Vainio, H. (1976). Action of styrene and its metabolites styrene oxide and styrene glycol on activities of xenobiotic biotransformation enzymes in rat liver *in vivo*. *Toxic. appl. Pharmac.* **38,** 59.

Compounds known to be converted to epoxides *in vivo* tend to be suspected of possible carcinogenic activity. This suspicion encouraged the mutagenicity studies on styrene and its metabolite, styrene oxide, described in the first paper cited above. *In vitro* studies were carried out on forward mutation systems of a yeast, *Schizosaccharomyces pombe*, and of Chinese hamster cells and on a gene-conversion system of *S. cerevisiae*. Also included were host-mediated assays, in which these yeasts were inoculated into the peritoneum of male Swiss mice treated by gavage with styrene or styrene oxide in a dose of 40 ml/kg.

Styrene oxide induced gene conversion in *S. cerevisiae* and forward mutation in *S. pombe* and hamster cells at concentrations as low as 5 mM, whereas styrene was inactive even at 100 mM. The latter result was not affected by conducting the incubations in the presence of mouse-liver microsomes, but the authors note that the activity of this metabolizing system was low, the conversion to styrene oxide being below 5%. In the host-mediated assay both compounds induced gene conversion but failed to increase forward-mutation frequency in *S. pombe*. It appears, therefore, that exposure to styrene vapour could lead to its conversion, in mammalian somatic cells, to the mutagenically active styrene oxide, although the present results were obtained with very high doses.

The second paper demonstrates that pretreatment of rats with three or six daily ip doses of 500 mg styrene/kg roughly doubled the activity of *p*-nitroanisole *O*-demethylase, and UDPglucuronosyltransferase in preparations of liver microsomes. The effect on UDPglucuronosyltransferase was only demonstrated in microsomal preparations pretreated *in vitro* with digitoxin or trypsin. Six doses of styrene increased NADPH-cytochrome *c* reductase by about 45% and cytochrome *P*-450 by 40%, but left aryl hydrocarbon hydroxylase almost unaffected. A single dose of 2 g styrene/kg or repeated daily injections of 500 mg/kg almost doubled epoxide hydratase activity. The amino-acid conjugation pathway was unaffected by three daily doses of 1 g styrene/kg. Styrene oxide was at least four to five times more toxic than either styrene or its glycol, and in a single dose of 375 mg/kg significantly reduced the cytochrome *P*-450 content and the microsomal activity of both aryl hydrocarbon hydroxylase and *p*-nitroanisole *O*-demethylase. Styrene oxide had little effect on epoxide hydratase, NADPH-cytochrome *c* reductase and UDPglucuronosyltransferase. The styrene-oxide derivative, styrene glycol, was almost inactive towards the drug-metabolizing enzyme systems. The enhancement of epoxide hydratase after styrene administration suggests that in chronic exposure the liver may increase its capacity to deal with metabolically produced styrene oxide.

3288. Revelations on dichloroacetylene neurotoxicity

Reichert, D., Liebaldt, G. & Henschler, D. (1976). Neurotoxic effects of dichloroacetylene. *Arch. Tox.* **37,** 23.

Dichloroacetylene (DCA) sometimes occurs as a decomposition product of trichloroethylene, tetrachloroethane or acetylene, or as a contaminant of some chlorinated monomers used in plastics production. Reports in the literature detailing experimental findings on the neurotoxic action of DCA are sparse. Humphrey & McClelland (*Br. med. J.* 1944, **I,** 315) reported briefly on numerous focal necroses with a generalized glial proliferation found in the brain, in association with an encephalitic reaction of the brain membranes, in two rabbits exposed to mixtures of trichloroethylene or ether and an unknown concentration of DCA. Additional work on mice revealed non-specific changes in various brain regions without pointing to the presence of distinct cranial lesions (Reichert *et al. Fd Cosmet. Toxicol.* 1975, **13,** 511). Intoxications in man have mainly been associated with signs of irreversible damage to the trigeminal nerve (Henschler *et al., Arch. Tox.* 1970, **26,** 62), although whenever selective lesions of this cranial nerve have been described in this connexion, the exposures have involved mixtures of DCA with other compounds.

The paper cited above outlines studies designed to clarify the extent and significance of the morphological and functional damage caused by DCA in the central nervous system of rabbits. The inhalation tests involved both lethal and sublethal doses of 126, 202 or 307 ppm DCA for 1 hr or 17 ppm for 6 hr. In the animals exposed to 202 ppm DCA for 1 hr, the most striking of the observed lesions were the cytological alterations affecting the sensory trigeminal nucleus followed (in order of decreasing intensity) by alterations to the nuclei of the facial, oculomotor, motor trigeminal and acoustic nerves. It was clear from histological study of brain sections from the group exposed to 307 ppm DCA for 1 hr that the pattern of neuropathological lesions was fully developed after 48 hr. Alterations in the temperature sensitivity of facial skin were demonstrated, showing that the trigeminal irritation threshold was significantly raised after DCA exposure.

While in man functional impairment of these cranial nerves has been found to be the main feature of DCA toxicosis, acute renal damage is the most prominent finding in animal experiments. Consideration of this aspect of the study was reserved for a subsequent communication. One purpose of the lowest dose (126 ppm for 1 hr) used in this work was to avoid uraemic effects and thus demonstrate more clearly the specific effects of DCA. At this concentration, cytological alterations were very much less

severe and consisted mainly of ganglion-cell pyknosis and hyperchromasia.

3289. A breath of formaldehyde

Hendrick, D. J. & Lane, D. J. (1977). Occupational formalin asthma. *Br. J. ind. Med.* **34,** 11.

Lancet (1977). Formalin asthma. *ibid* **I,** 790.

Formaldehyde (HCHO) has wide uses in industry, in dyeing, tanning, and the production of polymers for moulding and lacquering. Urea-formaldehyde condensates are used for cavity-wall insulation and may liberate formalin after insertion. Process workers curing phenol-formaldehyde resins have developed chronic airway obstructive disease (*Cited in F.C.T.* 1977, **15,** 84). Conventional industry is not the only place where exposure to HCHO occurs, for in hospitals it is commonly used as a tissue preservative, and more recently has been used for sterilizing Kiil artificial kidneys and pumping machines, so that repeated exposure of staff over many years may occur.

Of 28 staff members working in a dialysis unit, five reported that recurrent productive cough and wheezing, continuing for at least 3 yr, had affected them since they joined the unit. Three more people had suffered single episodes of the same symptoms during their exposure to HCHO. When given provocation tests with HCHO vapour, two of the first affected group developed wheezing 2–3 hr after exposure, with maximal reduction of peak expiratory flow rates of about 50% and a productive cough. Airways function remained impaired up to 10 days later. A threshold level of HCHO exposure was indicated by the fact that in one patient given provocation tests no reaction occurred until exposure to 25% HCHO had been extended from 5 to 15 min. Provocation testing of the other three in the asthmatic group failed to induce any pulmonary reaction, suggesting that in the two reactors the response was not non-specific. Sputum from affected patients was mucopurulent, but only a scanty growth of *Haemophilus influenzae* resulted from culture. This feature indicated the difficulty of distinguishing occupational asthma from bronchitis. The authors suggest that continual exposure to formalin vapour may increase susceptibility to attacks of bronchitis or induce a hyper-reactive response of the airways. Probably a relatively heavy exposure to HCHO is necessary to induce asthma, and well-designed premises for dialysis units can reduce the hazard.

Several other cases of respiratory illness as a result of formaldehyde exposure are cited in the *Lancet*. Formalin-induced asthma was reported in a laboratory technician, who had to give up his job as a result; and a neurologist involved in the preparation of brain specimens was very ill with hypoxia, pulmonary infiltrates and crackles audible over the lung bases.

3290. Phenyl-2-naphthylamine metabolized to 2-naphthylamine

Batten, P. L. & Hathway, D. E. (1977). Dephenylation of *N*-phenyl-2-naphthylamine in dogs and its possible oncogenic implications. *Br. J. Cancer* **35,** 342.

N-Phenyl-2-naphthylamine (PNA), an antioxidant used in the rubber industry, is not a known bladder carcinogen in either animals or man, unlike 2-naphthylamine (NA), which was previously used for the same purpose. However, the urine of PNA-exposed workers has recently been found to contain NA in greater quantities than could be explained by NA contamination of technical-grade PNA (Kummer & Tordoir, *Tijdschr. soc. Geneesk.* 1975, **53,** 415). The urine of 19 volunteers who each ingested 10 mg PNA (containing 0·008 μg NA) was in seven cases found to contain 0·4–3 μg NA in 24 hr, and four process operators estimated to have inhaled at least 40 mg PNA (containing 0·032 μg NA) excreted 3–8 μg NA in their urine. A study of PNA metabolism in the dog, which is the experimental animal most susceptible to bladder tumours from aromatic amines, has therefore been undertaken.

When adult dogs were given an oral capsule containing a 150-μCi dose of [^{14}C]PNA, more than 90% of the ^{14}C was eliminated within 3 days, principally via the bile and faeces. Not more than 2·8% appeared in the urine, even when the dogs had previously been given 400 mg unlabelled PNA on 5 days/wk for 4 wk. From a single oral dose of 5 mg unlabelled PNA/kg, dogs excreted on average 1·5 μg and at most 9·6 μg NA (measured as its more stable heptafluorobutyryl derivative). Great variability was evident between dogs, and in the output of any one dog at different times. NA excretion showed no consistent increase after continued dosing with PNA for 3 or 4 wk, suggesting that exposure to PNA does not induce microsomal enzymes involved in its degradation. No 2-amino-1-naphthyl sulphate (the principal metabolite of NA) or 2-naphthylhydroxylamine (suspected of being the ultimate carcinogenic derivative) could be detected in the urine. However, the analytical method used for the latter compound had a lower limit of detection of 50 ng, and small amounts may have escaped detection through decomposition.

On the basis of a dog study relating NA dose to the time taken for bladder tumours to develop (Conzelman & Moulton, *J. natn. Cancer Inst.* 1972, **49,** 193), it was estimated that the maximum of 9·6 μg NA excreted in the present study would take 19 years to produce tumours, a period appreciably more than the normal canine lifespan. The proportion of a PNA dose converted to NA was of about the same order in dogs as in man (Kummer & Tordoir, *loc. cit.*), although the dogs received more than ten times the human dose in terms of body weight. It is therefore considered probable that the levels of NA produced *in vivo* by PNA-exposed workers may be too small to represent any risk of carcinogenic effect during their lifespan.

[Notwithstanding these theoretical calculations, industrial handling practices designed to minimize exposure to *N*-phenyl-2-naphthylamine, such as those recommended by the US National Institute for Occupational Safety and Health (*Am. ind. Hyg. Ass. J.* 1977, **38,** A-21), would seem a wise precaution. A similar view has been taken by the American Conference of Governmental Industrial Hygienists in their

new classification of this compound as a suspected human carcinogen (ACGIH, *TLVs. Threshold Limit Values for Chemical Substances and Physical Agents in the Workroom Environment with Intended Changes for 1977*. p. 36. Cincinnati, OH).

3291. PCTs in the gut

Madge, D. S. (1977). Polychlorinated terphenyls and intestinal transport in mice. *Gen. Pharmac.* **8,** 43.

The distribution of polychlorinated biphenyls in the biosphere and their entry into food chains has received much attention (*Cited in F.C.T.* 1975, **13,** 574), and their metabolism in rodents, birds, fish and larger mammals has been described (*ibid* 1976, **14,** 349). The attention paid to the polychlorinated terphenyls (PCTs) has been more restricted (*ibid* 1974, **12,** 793), but this situation may soon change since it appears that they, too, may be widespread environmental pollutants.

Mice were given a single intragastric dose of Aroclor 5432 (32% Cl), Aroclor 5442 (42% Cl) or Aroclor 5460 (60% Cl), and examined 1 wk later. The materials, which were 99% pure, were given in doses ranging from 50 to 1000 mg/kg. Slight and inconclusive changes, including enlargement of the villi and distension of blood vessels, were observed in the small intestine of PCT-treated animals. Isolated small intestine showed a slight reduction in D-glucose absorption after 50- and 100-mg/kg doses, and a significant reduction after 250, 500 or 1000 mg/kg, with all three compounds. The absorption of D-galactose, L-arginine and L-histidine was not affected by PCT treatment. Serosal-fluid transfer and gut-fluid uptake of animals given any of the doses of PCTs did not differ significantly from those of controls. The addition of D-mannose to serosal fluid as an additional energy supply abolished any previous differences in D-glucose absorption. These results suggest that the effect of the PCTs is to alter the inherent endogenous energy of the absorptive cells of the small intestine rather than to interfere with the transport of D-glucose into the cells. In this respect the malabsorptive effects of PCTs closely resemble those previously described for some PCBs (Madge, *Gen. Pharmac.* 1976, 7, 45).

3292. Eye cancer—industrial causes?

Albert, D. M. & Puliafito, C. A. (1977). Choroidal melanoma: possible exposure to industrial toxins. *New Engl. J. Med.* **296,** 634.

Choroidal melanoma is an uncommon tumour in man. In view of the reported age-adjusted annual incidence of six per million population, the diagnosis of this type of tumour in two men working in a chemical plant in the Ohio Valley region was considered to merit further investigation. During the 3-yr period (1971–74) in which these tumours were detected, a third worker at the plant developed a squamous-cell carcinoma of the conjunctiva, and although no other eye tumours had been recorded at the plant in the period from 1956 to 1974, this brought the total number of observed cases of ocular cancer to six times the number that would have been expected in this population over the entire 18-yr period.

No common type of exposure could be identified for the three affected men, but three compounds used in the plant are considered to rank as possible human carcinogens. These are dimethyl sulphate, hydrazine and 4,4′-methylenedianiline. One worker, who died of generalized metastases from the choroidal melanoma, had been exposed to 25% aqueous hydrazine for 6 yr, while the second worker with melanoma had been exposed to dimethyl sulphate for 6 yr. This brief report suggests that the possible role of chemical agents in the aetiology of choroidal melanoma requires further investigation. In this context, it may be of interest that intra-ocular tumours have been reported in rats after oral administration of ethionine or *N*-2-fluorenylacetamide (Benson, *Archs Path.* 1962, **73,** 62) and after intra-ocular injection of methylcholanthrene (Patz *et al. Am. J. Ophthal.* 1959, **48,** 98), the latter tumour being a fibrosarcoma of the choroid.

NATURAL PRODUCTS

3293. Gourmet's guide to spirit congeners

Barnett, J. H. & Einsmann, J. R. (1977). Occurrence and distribution of 2-butanol, 1-butanol, 1-pentanol and 1-hexanol in distilled alcoholic beverages. *J. Ass. off. analyt. Chem.* **60,** 297.

There now seems to be little support for suggestions that the congeners that accompany ethanol in alcoholic beverages are more toxic than ethanol itself (*Cited in F.C.T.* 1975, **13,** 696). Nevertheless the determination of such congeners is a valuable means of discriminating between beverages and controlling their quality, and brief comment on a recent study seems appropriate.

Grape brandy contains 2-butanol, 1-butanol, 1-pentanol and 1-hexanol. These, together with acetaldehyde, methyl acetate, ethyl acetate, methanol, 1-propanol, isobutanol, isoamyl acetate, isoamyl alcohol, hexyl acetate, ethyl caprylate and 2-furfural were determined by gas–liquid chromatography in 198 brands of distilled alcoholic spirits available in Virginia in 1974 and 1975. The limit of detection of the first four alcohols by the method adopted was 0·1 g/100 litres.

1-Pentanol was present in concentrations exceeding 0·4 g/100 litres in only one brandy sample. Two of 70 brands of whisky contained 2-butanol at levels above 0·1 g/100 litres, but the concentration was not consistent within brands. All spirits except vodka contained 1-butanol, but generally not in sufficient variation to permit discrimination; in only one brand of whisky did the 1-butanol level exceed 0·6 g/100 litres. This alcohol was found to be a useful

distinguishing parameter for brands of tequila and grape brandy, however, and its highest concentration was achieved in apple brandy. 1-Hexanol occurred in all spirits except gin, and its highest concentrations (around 10 g/100 litres) occurred in apple brandy. In whisky, grape brandy and tequila, the variation in content of 1-hexanol was wide enough to form the basis of a distinction between different brands of the beverages, and its presence was consistent, making it the most useful index among the four congeners studied.

[While the chances of identifying a deletable constituent as a major factor in the toxicity of alcoholic liquors seems remote, chemistry shows some signs of eroding some of the mystique surrounding the potable spirits industry.]

3294. Lunchtime nips and blood-sugar levels

O'Keefe, S. J. D. & Marks, V. (1977). Lunchtime gin and tonic a cause of reactive hypoglycaemia. *Lancet* **I**, 1286.

Ethanol has been shown to inhibit glucose production in the body by shifting the $NAD^+/NADH$ ratio towards the reduced state, and this effect has been invoked to explain the hypoglycaemia readily produced in starved alcoholics (*Cited in F.C.T.* 1970, **8**, 436). The authors cited above show that the consumption of sugar in conjunction with ethanol tends to induce a delayed reactive hypoglycaemia which may be associated with impaired intellectual activity and judgement. This could contribute to motor-car accidents, particularly among late-afternoon drivers who drink at lunchtime but may not have excessive blood–alcohol levels at the time when their judgement is most impaired.

Pure glucose solutions given to normal subjects on an empty stomach may induce reactive hypoglycaemia, and when sweetened soft drinks are added to alcoholic spirits relatively large amounts of sugars may be ingested. In ten healthy young adults who drank on three separate occasions the equivalent of three gin and tonics containing 50 g ethanol and 60 g glucose, gin and carbohydrate-reduced tonics containing 50 g ethanol and 0·5 g sucrose, or tonic alone containing 60 g sucrose, behavioural effects, blood glucose and plasma insulin were monitored for 5 hr. Both ethanolic beverages caused mild to moderate inebriation, but the unsweetened tonic plus gin did not significantly alter the level of either blood glucose or plasma insulin. Gin plus sweetened tonic produced a greater insulinaemia and slightly but not significantly greater initial hyperglycaemia than did tonic alone. However there was a significantly greater hypoglycaemic rebound after the gin and tonic and in three subjects the effect was great enough to elicit signs of nervous system disturbance. The blood-glucose levels were lowest after about 3 hr.

[The number of subjects used in this study was low but in view of their implications, these findings seem worthy of further investigation.]

3295. Tyrosine toxicity analysed

David, J. C. (1976). Evidence for the possible formation of a toxic tyrosine metabolite by the liver microsomal drug metabolizing system. *Naunyn-Schmiedeberg's Archs Pharmac.* **292**, 79.

Ingestion of excessive amounts of tyrosine by animals can produce various toxic manifestations, including growth depression and eye and paw lesions, but the mechanism has not been demonstrated. It has been shown that reduced food intake is not the main cause of tyrosine toxicity, and a lack of toxic effects in the eyes and paws of animals fed the tyrosine metabolites *p*-hydroxyphenylpyruvic acid and dopaquinone has indicated that the parent amino acid may be the primary toxic agent (*Cited in F.C.T.* 1971, **9**, 137 & 158). It is now suggested, however, that the effect of tyrosine on the liver, an important target organ, may depend on its conversion to a toxic metabolite by the cytochrome *P*-450 system of the microsomal fraction.

Groups of mice were pretreated with various microsomal-enzyme inducers or inhibitors prior to receiving two equal oral doses of tyrosine, in the range 0·5–3·5 g/kg, separated by a 7-hr interval. A decarboxylase inhibitor, N_1-(DL-seryl)-N_2-(2,3,4-dihydroxybenzylhydrazine), was injected ip in a dose of 50 mg/kg when each tyrosine dose was given, to depress metabolic decarboxylation of the tyrosine to tyramine and thus to reduce the dose of tyrosine required to elicit toxic effects, making comparative LD_{50} studies a practical possibility. The alternative pretreatments were all given ip and consisted either of one of the microsomal-enzyme inducers phenobarbitone (80 mg/kg) and 3-methylcholanthrene (50 mg/kg), given in each case on four consecutive days, the last treatment being 22 hr before the first dose of tyrosine, or of one of two inhibitors, the microsomal-system inhibitor SKF 525A (a single dose of 50 mg/kg given 1 hr before the tyrosine) and cobaltous chloride ($CoCl_2$), an inhibitor of cytochrome *P*-450 synthesis, given in two doses of 60 mg/kg on consecutive days, the second dose 22 hr before the first tyrosine dose.

Inhibition of the microsomal cytochrome *P*-450 system with either SKF 525A or $CoCl_2$ markedly reduced the acute toxicity of tyrosine, whereas pretreatment with one of the inducers, phenobarbitone or 3-methylcholanthrene, potentiated it. The findings are thus consistent with the view that the toxicity of tyrosine is likely to be due to its conversion to a toxic metabolite by the microsomal-enzyme system. Further studies involving ip treatment of mice with either diethyl maleate (100 mg/kg) or cysteine (50 mg/kg) 1 hr before the tyrosine/carboxylase inhibitor treatment showed that tyrosine toxicity correlated with a depletion in the glutathione (GSH) content of the liver and suggested that the toxic metabolite could be a tyrosine-2,3-epoxide. Thus toxic doses of tyrosine were found to lower the liver-GSH level, and pretreatment with diethyl maleate, which reduces the liver-GSH content by depressing the availability of

endogenous GSH, potentiated tyrosine toxicity. Cysteine, on the other hand, maintained tissue levels of GSH and was found to reduce the toxicity of tyrosine.

Further support for the postulated formation of an epoxide intermediate *in vivo* can be found in clinical reports of urinary excretion products associated with cases of tyrosinaemia, but other possible metabolites cannot be altogether excluded.

As a corollary to these findings, the author of this paper recommends that patients who suffer from tyrosinaemia ought not to receive microsomal-enzyme inducers.

COSMETICS, TOILETRIES AND HOUSEHOLD PRODUCTS

3296. *In vitro* evidence of mutagenesis by a 2,4-diaminoanisole metabolite

Dybing, E. & Thorgeirsson, S. S. (1977). Metabolic activation of 2,4-diaminoanisole, a hair-dye component—I. Role of cytochrome P-450 metabolism in mutagenicity *in vitro*. *Biochem. Pharmac.* **26**, 729.

Oxidation-hair-dye formulations may include 2,4-diaminoanisole. It has been reported that no toxic or carcinogenic effects were detected in mice treated topically with such preparations (Burnett *et al. Fd Cosmet. Toxicol.* 1975, **13**, 353), and another skin-painting study in mice was also negative in respect of carcinogenic activity (Giles *et al. J. Toxicol. envir. Hlth* 1976, **1**, 433). Further, and more positive, information about the 2,4-diaminoanisole component is now available but the *in vivo* significance of the results of these *in vitro* studies remains a very open question.

Mutation experiments were performed with liver fractions prepared from rats and mice pretreated with ip or sc injections of phenobarbitone, β-naphthoflavone, Aroclor 1254, cobaltous chloride ($CoCl_2$) or piperonyl butoxide to induce or inhibit the cytochrome *P*-450 enzyme system. The various liver fractions and 2,4-diaminoanisole were then incubated with Salmonella of strain TA1538 in a series of Ames-type tests (*Cited in F.C.T.* 1976, **14**, 353).

Pretreatment of both rats and mice with either of the enzyme inducers phenobarbitone and β-naphthoflavone increased mutation rates in the test organism, the second compound being several times more active than the first in this respect. $CoCl_2$, which inhibits cytochrome *P*-450 synthesis, reduced the subsequent mutation rate when given to rats, but not to mice. Somewhat anomalously, piperonyl butoxide, an inhibitor of many reactions mediated by the *P*-450 system, increased the mutation rate when given to either species, a possible indication of preferential inhibition of a non-mutagenic pathway and a consequent increase in the substrate available for a mutagenic pathway. Aroclor 1254 pretreatment, which effectively increased the mutagenic activity of 2,4-diaminoanisole incubated with liver fractions, had a less marked effect on the ability of kidney and lung fractions to convert 2,4-diaminoanisole into an active mutagen for Salmonella TA1538. An increase in mutagenic activity was also recorded in incubations of 2,4-diaminoanisole and enzyme preparations of foetal liver, when the pregnant rats had been treated with β-naphthoflavone. Most of these studies were carried out with the 9000-g supernatant (S9) fractions of liver and other tissues. On further fractionation, most of the activity was found in the microsomes, little being detected in the 105,000-g supernatant.

2,4-Diaminoanisole was by far the most active mutagen among a group of ten aryldiamines tested by the method outlined. The revertant rate it induced was not markedly altered by pretreatment of the animals with diethyl maleate or cysteine, to deplete or maintain liver-glutathione levels, or by direct addition of diethyl maleate to the test plate. The authors suggest that the apparent activation of 2,4-diaminoanisole to a potential mutagen by the cytochrome *P*-450 system could possibly involve the formation of an intermediate *N*-hydroxylated metabolite.

Fd Cosmet. Toxicol. Vol. 16, p. 305. Pergamon Press 1978. Printed in Great Britain

MEETING ANNOUNCEMENTS

ASPET–SOT MEETING IN TEXAS

The Fall Meeting of the American Society for Pharmacology and Experimental Therapeutics and the Society of Toxicology will be held at the University of Houston, Houston, Texas, on 13–17 August 1978.

In addition to a number of scientific sessions, there will be symposia concerned specifically with the passage of substances across membranes, the quantification of drugs in biological fluids, drug metabolism and the safety of xenobiotics, tachyphylaxis to catecholamines, cancer chemotherapy, and the role of epidemiology in toxicology.

Further information may be obtained from: ASPET–SOT, College of Pharmacy 141 SR-2, University of Houston, Houston, TX 77004, USA.

STERILIZATION SYMPOSIUM

The Laboratory of Cosmetology and Industrial Pharmacy of the University of Nantes has announced a symposium, to be held at Nantes on 24–26 October 1978, concerned with sterilization processes involving the use of ethylene oxide or irradiation. Sterilization of foods, drugs, medico-surgical materials, cosmetics and packaging will be discussed from the points of view of equipment, facilities, dosage and other conditions, precautions and existing regulations. Further information may be obtained from: Madame le Professeur Y. de Roeck-Holtzhauer, Laboratoire de Cosmétologie et de Pharmacie Industrielle, 68, Boulevard Eugène Orieux, 44300 Nantes, France; tel no. (40) 74.23.22/(40) 74.32.89.

Fd Cosmet. Toxicol. Vol. 16. p. 306. Pergamon Press 1978. 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:*

Gut microflora interactions with two experimental polymeric food additives in the rat. By J. P. Brown, R. J. Brown, B. C. Hyde and C. M. Bakner.

Intestinal absorption, distribution and excretion of an orally administered polymeric antioxidant in rats and mice. By T. M. Parkinson, T. Honohan, F. E. Enderlin, S. C. Halladay, R. L. Hale, S. A. de Keczer, P. L. Dubin, B. A. Ryerson and A. R. Read.

Studies on the purity and short-term toxicity of ethyl methylphenylglycidate (strawberry aldehyde) in rats. By P. L. Mason, K. R. Butterworth, I. F. Gaunt, P. Grasso and S. D. Gangolli.

Short-term toxicity study of isobutyl isobutyrate in rats. By J. J.-P. Drake, K. R. Butterworth, I. F. Gaunt and P. Grasso.

Occurrence of *N*-nitrosamino acids in cured meat products and their effect on formation of *N*-nitrosamines during heating. By C. Janzowski, G. Eisenbrand and R. Preussmann.

The metabolism of buturon in the rat. By W. Grunow, H.-J. Altmann und Chr. Böhme.

Citrinin mycotoxicosis in the Syrian hamster. By W. H. Jordan, W. W. Carlton and G. A. Sansing.

Comparison of the efficiencies of ascorbic acid and sulphamic acid as nitrite traps. By D. L. H. Williams. (Short paper)

Acute toxicity studies on roquefortine and PR toxin, metabolites of *Penicillium roqueforti*, in the mouse. By D. L. Arnold, P. M. Scott, P. F. McGuire, J. Harwig and E. A. Nera. (Short paper)

An appeal for reason. By H. B. Morley. (Review paper)