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*These items have been contributed by the staff of the British Industrial Biological Research Association. Comments on them will be welcomed and should be sent to the Assistant Editor at BIBRA.

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Research Section

LONG-TERM TOXICITY STUDIES OF CARMOISINE IN MICE

P. L. MASON, I. F. GAUNT, K. R. BUTTERWORTH, JOAN HARDY, IDA S. KISS and P. GRASSO

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(*Received 8 March 1974*)

Abstract—Carmoisine was fed to mice at dietary levels of 0 (control), 0·01, 0·05, 0·25 or 1·25% for 80 wk. There were no adverse effects on mortality, weight gain, organ weights or the incidence of histopathological findings, including tumours. There was a mild anaemia in the mice given 1·25% carmoisine. It is concluded that carmoisine has no carcinogenic potential in mice at dietary levels up to 1·25% and that the no-untoward-effect level is 0·25% (equivalent to an intake of approximately 350 mg/kg/day).

INTRODUCTION

The principal constituent of carmoisine (C.I. (1971) no. 14720; EEC no. E122) is the disodium salt of 2-(4-sulpho-1-naphthylazo)-1-naphthol-4-sulphonic acid (also known as disodium 4-hydroxy-3-(4-sulpho-1-naphthylazo)naphthalene-1-sulphonate). It is one of the colourings permitted for use in the UK under the Colouring Matter in Food Regulations 1966 (Statutory Instrument 1966, no. 1203) and its use may continue after July 1974 under the Colouring Matter in Food Regulations 1973 (Statutory Instrument 1973, no. 1340). Carmoisine is also permitted for use in food in the EEC countries, but in the USA its use is not allowed.

The view that the available evidence on its safety-in-use was inadequate was expressed by both the Food Standards Committee (1964) in its Report on Colouring Matters and the Joint FAO/WHO Expert Committee on Food Additives (1965).

The acute toxicity has been investigated in both rats and mice. After ip injection, the LD_{50} was 1·0 g/kg in rats and 0·9 g/kg in mice (Gaunt, Farmer, Grasso & Gangolli, 1967). The LD_{50} after iv injection in mice was 0·8 g/kg (Hecht, unpublished data, cited by Deutsche Forschungsgemeinschaft–Farbstoff-Kommission, 1957). However, oral doses of 10 g/kg in the rat and 8 g/kg in the mouse were tolerated without effect (Gaunt *et al.* 1967). A similar lack of toxic effect after oral administration has been found in short-term studies. Carmoisine was fed to rats at dietary levels of 0·05, 0·1, 0·5 and 1·0% of the diet for 90 days. The only effect observed was an increase in relative kidney weight in females at the 1% dietary level, and a no-effect level of 0·5% was established (Gaunt *et al.* 1967). A no-effect level of 1 g/kg/day was found in miniature pigs given carmoisine at dose levels of 250, 500 and 1000 mg/kg/day for 90 days (Gaunt, Grasso, Kiss & Gangolli, 1969).

Long-term studies using groups of ten rats have been reported (Deutsche Forschungsgemeinschaft–Farbstoff-Kommission, 1957). These included administration of a diet containing 0·2% carmoisine for 417 days with observation for up to 838 days, and two studies

in which 1·0% was given in the drinking-water for 209 and 250 days with observation for 919 and 545 days respectively. No tumours were seen in these animals. One spindle-cell sarcoma was found in a group of ten rats given twice-weekly sc injections of 0·5 ml of a 1·0% solution of carmoisine for 365 days with subsequent observation for up to 938 days. However, no tumours were produced in a second similar experiment with observation for 521 days. In mice, Bonser, Clayson & Jull (1956) found only one benign intestinal polyp in 30 mice given twice-weekly sc injections of 0·1 ml of a 3–6% suspension of carmoisine in water for 360 days with subsequent observation for periods in excess of 630 days.

The following paper presents the results of an 80-wk feeding study in male and female mice. The work was undertaken as part of the BIBRA safety evaluation programme, to provide more information on the long-term toxicity of carmoisine.

EXPERIMENTAL

The carmoisine used in this study was supplied through the Food Colours Committee of the Chemical Industries Association and complied with the following specification of the British Standards Institution (1961):

> Carmoisine consists essentially of the disodium salt of 2-(4-sulpho-1-naphthylazo)-1-naphthol-4-sulphonic acid. Dye content, min 85%; matter volatile at 135°C, max 10%; matter insoluble in water, max 0·1%; matter soluble in diisopropyl ether, max 2·0%; subsidiary dyes, max 2·0%; chlorides and sulphates (as sodium salts), max 5·0%; copper, max 10 ppm; arsenic, max 1 ppm; lead, max 10 ppm; heavy metals (as sulphides) not producing more intense colour than the reference standard.

Animals and diet. Male and female mice of the ASH/CS1 strain were used. They were given free access to Oxoid pasteurized breeding diet supplemented with 80 ppm vitamin K_3 and to water. The animals were housed in a room maintained at 21 ± 1°C with a relative humidity of 50–60%.

Experimental design and conduct. Groups of 30 male and 30 female mice were fed diets containing 0·01, 0·05, 0·25 or 1·25% carmoisine for 80 wk. The control group consisted of 60 males and 60 females fed the same basic diet for 80 wk. The mice were housed in cages of five and were weighed every 2 wk up to wk 57 and at the end of the study. Haematological analyses were carried out on ten animals from the control group and those given the two highest dietary levels (0·25 and 1·25%) at wk 12, 24 and 52, using blood collected from a tail vein. The blood was examined for haemoglobin concentration and packed cell volume, and the numbers of erythrocytes and leucocytes were counted. In addition, differential leucocyte and reticulocyte counts were made on the blood samples from the mice fed the control diet and those fed the highest level of carmoisine. At the end of the study the blood samples from all surviving animals were examined for haemoglobin and differential leucocytes only.

The animals were kept under continual surveillance for any abnormalities in condition or behaviour and those that became ill were killed if they failed to show signs of recovery. An autopsy was carried out on all animals killed or found dead during the experiment, unless this was precluded by advanced autolysis or cannibalism.

At the end of the study the remaining animals were killed by exsanguination from the aorta under barbiturate anaesthesia following an overnight period without food. An autopsy was conducted at which any macroscopic abnormalities were noted and the brain,

heart, liver, spleen and kidneys were weighed. Samples of these tissues, together with salivary gland, thyroid, adrenal glands, lymph nodes, pancreas, pituitary, ovaries, uterus, urinary bladder, trachea, lungs, oesophagus, stomach, small intestine, colon, rectum, spinal cord and skeletal muscle and any other tissue that appeared to be abnormal, were preserved in 10% buffered formalin. Paraffin-wax sections of these tissues were stained with haematoxylin and eosin. All tissues from the mice fed control diet and those fed 1·25% carmoisine were examined microscopically, while at the lower dietary levels examination was confined to the heart, liver and kidney together with any tissue that appeared to be abnormal at autopsy.

RESULTS

There was external contamination of the fur by the coloured diet. The faeces in the mice given dietary levels of 0·25 or 1·25% were normally coloured when voided, but rapidly (in 1–2 min) darkened to a purple colour on exposure to the air. There was some red colouring of the urine at most dose levels but samples taken with precautions against contamination showed no red colour, although at the 1·25% level there was a slight brownish colour. No haemoglobin or red cells were present in the urine.

The ingestion of carmoisine had no effect on either the condition or the behaviour of the animals. Although deaths occurred in all treatment groups during the study, there was no association between the death rate or the total number of animals dying and the presence of carmoisine in the diet. Thus at wk 50, the cumulative deaths in groups of 60 control mice of each sex were 7 males and 6 females, compared with 6, 3, 3 and 6 males and 5, 3, 3 and 7 females in groups of 30 fed carmoisine at levels of 0·01, 0·05, 0·25 and 1·25%, respectively. Deaths by wk 80 totalled 22 male and 23 female controls and 10, 10, 15 and 8 males and 8, 13, 7 and 16 females in the corresponding test groups. Similarly, carmoisine was not found to have any adverse effect on the rate of body-weight gain, the mean weight gain at wk 57 being 19, 18, 17 and 17 g in males and 18, 19, 19 and 15 g in females fed 0·01, 0·05, 0·25 and 1·25% carmoisine, respectively, compared with a mean gain of 18 g in controls of both sexes.

There were no differences in absolute or relative organ weights, between the treated and control mice (Table 1). In female mice given 1·25% carmoisine, the haemoglobin concentration was significantly lower than the controls after treatment for 12 and 52 wk (Table 2). In male mice, the same effect was seen at wk 52 with dietary levels of 0·25 and 1·25%. In both sexes given 1·25% carmoisine for 12 wk, this finding was associated with a lower reticulocyte count and at wk 52 the packed cell volume was lower in the females. The only significant difference in the leucocytes was a reduced total count in the females given the highest dietary level of carmoisine for 24 wk.

At autopsy the contents of the small intestine, but not the caecum or colon, were coloured red in mice given 0·25 or 1·25% carmoisine. On exposure to air the contents of the caecum and colon became purple-coloured.

The histopathological changes were those normally encountered in mice of this age and the incidence was similar in all treatment groups within each sex (Table 3). The tumours found are shown in Table 4. Pulmonary adenomas were present in both male and female groups in similar numbers. A hepatocellular adenoma and a subcutaneous fibroma occurred in two male control mice and a single granulosa-cell tumour was found in a

Table 1. *Relative organ weights of mice fed carmoisine at 0–1·25% in the diet for 80 wk*

Dietary level (%)	No. of mice examined	Relative organ weight (g/100 g body weight) Brain	Heart	Liver	Spleen	Kidney	Terminal body weight (g)
		Males					
0	32	1·31	0·73	5·32	0·46	1·92	36
0·01	20	1·22	0·70	5·40	0·58	1·92	38
0·05	18	1·20	0·68	4·94	0·44	1·83	37
0·25	13	1·21	0·72	5·48	0·55	1·88	37
1·25	22	1·29	0·68	5·22	0·47	2·04	36
		Females					
0	35	1·37	0·58	5·47	0·85	1·56	33
0·01	19	1·40	0·61	5·47	0·82	1·49	33
0·05	15	1·69	0·63	5·36	0·81	1·53	33
0·25	22	1·37	0·58	5·30	1·10	1·51	34
1·25	9	1·53	0·68	5·09	0·90	1·58	29

Values are means for the numbers of mice shown.

Table 2. *Haematological values in mice fed carmoisine at 0–1·25% in the diet for up to 80 wk*

Sex and dietary level (%)	No. of mice examined	Hb (g/100 ml)	PCV (%)	RBC ($10^6/mm^3$)	Retics (% RBC)	Leucocytes Total ($10^3/mm^3$)	Differential (%) N	E	L	M
				Wk 52						
Male										
0	10	14·6	48	8·87	2·20	10·7	31	0	68	0
0·25	10	11·2***	45	7·83	—	10·4	—	—	—	—
1·25	10	12·2***	46	8·53	1·57	10·8	36	0	63	0
Female										
0	10	15·7	48	8·87	2·29	10·8	28	0	71	0
0·25	10	15·3	48	8·33	—	10·0	—	—	—	—
1·25	10	13·0***	44*	7·83	1·89	6·5	32	0	67	0
				Wk 80						
Male										
0	31	14·2	—	—	—	—	45	0	55	0
0·01	19	14·2	—	—	—	—	—	—	—	—
0·05	17	15·3	—	—	—	—	—	—	—	—
0·025	12	14·0	—	—	—	—	—	—	—	—
1·25	19	14·8	—	—	—	—	39	0	61	0
Female										
0	35	15·2	—	—	—	—	43	0	56	1
0·01	20	14·5	—	—	—	—	—	—	—	—
0·05	13	14·5	—	—	—	—	—	—	—	—
0·25	22	15·1	—	—	—	—	—	—	—	—
1·25	9	13·5	—	—	—	—	45	0	55	0

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

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

female control. There were three renal adenomas in male mice, one in each of the groups fed 0 (control), 0·01 and 0·25% carmoisine. Several malignant tumours occurred in both male and female mice. Two fibrosarcomas and a mammary adenocarcinoma were found in two male mice fed control diet and one female mouse fed 1·25% carmoisine, respectively. Neoplasms of the reticulo-endothelial system included lymphomas in two male controls and a lymphosarcoma in a male mouse fed 0·25% carmoisine. Lymphoblastomas occurred in both male and female mice, with a similar incidence in all groups.

Table 3. *Incidence of histopathological findings in mice fed carmoisine at 0–1·25% of the diet for 80 wk*

Organ and finding	Incidence of histopathological finding in: Males fed dietary levels (%) of					Females fed dietary levels (%) of				
	0	0·01	0·05	0·25	1·25	0	0·01	0·05	0·25	1·25
No. of mice examined...	48	26	26	24	28	55	23	26	27	19
Liver										
Necrotic areas	11	3	7	2	5	3	1	2	0	2
Amyloid deposits	3	1	0	1	0	1	0	0	0	0
Fatty change	3	0	0	0	2	7	2	0	0	1
Nodular hyperplasia	6	1	0	0	2	0	0	0	0	0
Foamy macrophages	0	0	0	1	0	0	0	0	0	0
Lymphoid hyperplasia	0	1	0	1	0	0	0	0	2	0
Kidneys										
Perivascular lymphocytes	16	6	1	1	4	14	7	6	2	2
Pyelonephritis	7	1	0	2	1	5	2	2	0	1
Tubular casts	1	0	0	0	0	4	1	2	0	0
Lymphoid hyperplasia	0	4	1	2	0	1	1	0	1	0
Spleen										
Amyloid deposits	2	1	0	1	0	2	1	0	0	0
Lymphoid hyperplasia	2	4	2	3	0	3	3	0	2	1
Lungs										
Peribronchial lymphocytes	0	0	0	0	0	0	0	0	1	0
Collapse	0	0	0	1	0	0	0	0	0	0
Bladder										
Chronic inflammation	2	0	0	0	2	0	1	0	0	0
Cowpers gland										
Chronic inflammation	0	0	1	0	1	—	—	—	—	—
Caecum										
Lymphoid hyperplasia	0	0	0	0	0	2	3	0	2	0
Skin										
Chronic inflammation	1	0	0	0	1	0	0	0	0	0
Testes										
Atrophy	0	0	0	1	0	—	—	—	—	—
Metaplasia	0	0	0	0	1	—	—	—	—	—
Ovaries										
Cysts	—	—	—	—	—	0	0	2	0	0
Thymus										
Lymphoid hyperplasia	0	0	0	0	0	5	1	0	1	3
Pituitary										
Haemorrhagic cyst	0	1	0	0	0	0	0	0	0	0
Adrenals										
Amyloid deposists	1	0	0	1	0	0	0	0	0	0

The figures represent the incidence of histopathological findings in the number of mice shown.

Table 4. *Incidence of tumours in mice fed carmoisine at 0–1·25% of the diet for 80 wk*

Organ and type of tumour	No. of mice examined...	Males 0	Males 0·01	Males 0·05	Males 0·25	Males 1·25	Females 0	Females 0·01	Females 0·05	Females 0·25	Females 1·25
		48	26	26	24	28	55	23	26	27	19
Lung											
Papillary adenoma		13	5	7	4	8	4	2	4	4	2
Liver											
Adenoma		1	0	0	0	0	0	0	0	0	0
Kidneys											
Adenoma		1	1	0	1	0	0	0	0	0	0
Subcutaneous tissue											
Fibroma		1	0	0	0	0	0	0	0	0	0
Fibrosarcoma		2	0	0	0	0	0	0	0	0	0
Ovaries											
Granulosa-cell tumour		—	—	—	—	—	1	0	0	0	0
Mammary gland											
Adenocarcinoma		—	—	—	—	—	0	0	0	0	1
Reticuloendothelial system											
Lymphoma		2	0	0	0	0	0	0	0	0	0
Lymphosarcoma		0	0	0	1	0	0	0	0	0	0
Lymphoblastoma		2	2	2	1	1	10	4	3	1	3

Column groups: Incidence of tumour in Males fed dietary levels (%) of / Females fed dietary levels (%) of.

The figures represent the numbers of mice affected.

DISCUSSION

In this study no adverse effects were detected in mice fed dietary levels of 0·01 or 0·05% carmoisine for the greater part of their life. The observation that the contents of the small intestine were coloured red, whereas those of the caecum and large intestine were not so coloured, suggested either that the bulk of the colouring was absorbed from the small intestine or that it was metabolized in the lower gut. The former seems unlikely, since the intensity of the colouring did not decrease in the lower portions of the small intestine but showed a distinct change in the region of the ileo-caecal junction. In addition, Ryan & Wright (1961) showed that the majority of an iv dose of the colouring was excreted unchanged in the bile and so, if absorbed unchanged, would be returned to the gastro-intestinal tract. However, no colouring of the bile was seen in the present study. Thus it seems likely that some metabolism occurred in the caecum, as has been shown with many other azo colours (Walker, 1970). The observation that the faeces and the contents of the caecum and colon changed from a normal colour to purple on exposure to the air indicated that some colourless metabolite was produced in the lower gut and on exposure to the air this was converted to a purple-coloured material. The brownish colour of the urine of mice given 1·25% carmoisine may represent absorbed metabolites undergoing excretion. Clearly further investigations on the absorption, metabolism and excretion of carmoisine are required to clarify these observations.

During the first year of study there were reductions in haemoglobin concentration in animals fed 1·25% carmoisine, although the differences were not always statistically significant. At the end of the study the mean haemoglobin concentration was not significantly affected, although the number of mice that might be considered anaemic (haemoglobin 11·0 g/100 ml) was significantly greater than in the control animals (chi-square, $P < 0·05$).

Examination of the blood films showed some hypochromasia and anisocytosis but no intra-erythrocytic inclusions were found in fixed or vitally stained smears that were not similarly present in the control animals. In addition, mice given 1·25% carmoisine for 12 wk exhibited reduced reticulocyte counts, suggesting that the anaemia may have been related to some defect in erythropoiesis rather than to an increased destruction of red cells, as the latter is normally accompanied by an increased erythropoiesis and reticulocyte count. Animals given 0·25% carmoisine for 52 wk showed a mild anaemia, but this isolated finding may well have been random. However, until evidence to the contrary is produced, the anaemia in mice given 1·25% carmoisine must be regarded as an effect of the colouring. Although this effect was seen after wk 12, no similar effects have been reported in short-term studies in rats (Gaunt *et al.* 1967) or pigs (Gaunt *et al.* 1969) suggesting that this may be an effect peculiar to the mouse.

There was no identification of any toxic effect of carmoisine in the histopathological examination and most of the tumours occurred with a similar frequency in treated and control mice or in controls alone. Tumours occurring in treated animals only were a mammary adenocarcinoma in a female given 1·25% and a lymphosarcoma in a male given 0·25% carmoisine. However, mammary carcinoma has been reported to occur in untreated mice (Tucker & Baker, 1967) and has been found in these laboratories in control mice in other studies (Gaunt, Brantom, Grasso & Kiss, 1973). Similarly lymphosarcoma both of the thymus and generalized (Tucker & Baker, 1967) has been reported in untreated mice. Thus the isolated findings of these two tumours cannot be taken to indicate any carcinogenic effect on the part of carmoisine. It is concluded therefore that carmoisine showed no carcinogenic effect when given to mice at dietary levels of up to 1·25% (equivalent to an intake of approximately 1800–2000 mg/kg body weight/day).

In these studies the highest level causing no observable effect was 0·05%; at a dietary level of 1·25% there was an indication of a toxic effect in the form of a mild anaemia. However, at the intermediate level of 0·25% the changes seen were of doubtful significance and it is considered that this represents the true no-untoward-effect level. At 0·25% of the diet the daily intake of colouring was approximately 350 mg/kg. After application of the usual 100-fold safety factor, this suggests an acceptable daily intake of 210 mg for a 60-kg adult.

REFERENCES

Bonser, Georgiana M., Clayson, D. B. & Jull, J. W. (1956). The induction of tumours of the subcutaneous tissues, liver and intestine in the mouse by certain dyestuffs and their intermediates. *Br. J. Cancer* **10**, 653.

British Standards Institution (1961). Specification for Carmoisine for Use in Foodstuffs. B.S. 3343:1961.

Deutsche Forschungsgemeinschaft Farbstoff-Kommission (1957). Mitteilung 6, 2. Auflage. *Toxikologische Daten von Farbstoffen und ihre Zulassung für Lebensmittel in verschiedenen Ländern*, p. 38. Franz Steiner Verlag GmbH, Wiesbaden.

Food Standard Committee (1964). Report on Colouring Matters. HMSO, London.

Gaunt, I. F., Brantom, P. G., Grasso, P. & Kiss, Ida S. (1973). Long-term toxicity studies of Chocolate Brown FB in mice. *Fd Cosmet. Toxicol.* **11**, 375.

Gaunt, I. F., Farmer, Madge, Grasso, P. & Gangolli, S. D. (1967). Acute (mouse and rat) and short-term (rat) toxicity studies on carmoisine. *Fd Cosmet. Toxicol.* **5**, 179.

Gaunt, I. F., Grasso, P., Kiss, Ida S. & Gangolli, S. D. (1969). Short-term toxicity of carmoisine in the miniature pig. *Fd Cosmet. Toxicol.* **7**, 1.

Joint FAO/WHO Expert Committee on Food Additives—Eighth Report (1965). Specifications for the Identity and Purity of Food Additives and their Toxicological Evaluation: Food Colours and Antimicrobials and Antioxidants. *Tech. Rep. Ser. Wld Hlth Org.* **309**.

Ryan, A. J. & Wright, S. E. (1961). The excretion of some azo dyes in rat bile. *J. Pharm. Pharmac.* **13**, 492.

Tucker, Mary J. & Baker, S. B. de C. (1967). Diseases of specific pathogen-free mice. In *Pathology of Laboratory Rats and Mice*. Edited by E. Cotchin & F. J. C. Roe. p. 799. Blackwell Scientific Publications, Oxford.

Walker, R. (1970). The metabolism of azo compounds: A review of the literature. *Fd Cosmet. Toxicol.* **8**, 659.

Fd Cosmet. Toxicol. Vol. 12 pp. 609-624. Pergamon Press 1974. Printed in Great Britain

SHORT-TERM TOXICITY OF CYCLOHEXYLAMINE HYDROCHLORIDE IN THE RAT

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Abstract—Groups of 15 male and 15 female rats were given diets containing 0 (control), 600, 2000 or 6000 ppm cyclohexylamine hydrochloride (CHA) for 13 wk. No changes in the haematological examinations, serum analyses or urinary cell excretion values were associated with CHA treatment. At the two higher dosage levels there was a reduced rate of body-weight gain and food intake. Paired feeding showed that the reduced food intake did not fully account for the failure to gain weight. The absorption of nutrients was normal but measurements of oxygen consumption suggested that an increased basal metabolic rate might account, in part, for the failure to gain weight. The renal concentrating ability was slightly reduced in the females given the highest dietary concentration of CHA. The weights of most of the organs were lower in the rats given diets containing 2000 or 6000 ppm CHA but this was related to the reduced body weight. The testis weight and relative testis weight were reduced and histopathological examination showed reduced spermatogenesis. However, the rats remained fertile and there was no evidence of abnormalities in their offspring. Apart from the effects on the testis, no changes were seen in the histopathological examination. The no-untoward-effect level established in this study was 600 ppm, a level equivalent to an intake of approximately 30 mg CHA/kg/day.

INTRODUCTION

Cyclohexylamine (CHA) is a strongly basic compound which readily forms salts with acids, including carbon dioxide and fatty acids (Carswell & Morrill, 1937). CHA and its derivatives are used industrially as corrosion inhibitors and in the vulcanizing of rubber, but the present toxicological interest in CHA centres on its occurrence as a metabolite of the cyclamate sweeteners, the sodium and calcium salts of cyclohexylsulphamate.

Extensive studies have been carried out on the effects of cyclamate in animals and man and these have included investigation of its metabolism. Early studies indicated that cyclamates were quantitatively excreted unchanged in the faeces and urine (Miller, Crawford, Sonders & Cardinal, 1966; Schoenberger, Rix, Sakamoto, Taylor & Kark, 1953; Taylor, Richards & Davin, 1951). However, Kojima & Ichibagase (1966) reported that, in four human subjects, approximately 0·7% of an oral dose of 1·0 g cyclamate was converted to CHA within 24 hr. This conversion in man has been confirmed by a number of workers (A. J. Collings, Unilever Research Laboratory, unpublished data 1971; Leahy, Taylor & Rudd, 1967; Leahy, Wakefield & Taylor, 1967; Renwick & Williams, 1972b). Conversion of up to 60% of the dose of cyclamate has been reported (A. J. Collings, unpublished data 1971). In addition to CHA, Golberg, Parekh, Patti & Soike (1969) found cyclohexanone, cyclohexanol and *N*-hydroxycyclohexylamine as minor metabolites in volunteers given cyclamate.

Conversion of cyclamate to CHA has also been demonstrated in dogs (Golberg *et al.* 1969; Kojima & Ichibagase, 1966), rats (Oser, Carson, Vogin & Sonders, 1968), rabbits

and guinea-pigs (Renwick & Williams, 1972b), pigs (A. J. Collings, unpublished data 1971) and rhesus monkey (Parekh, Goldberg & Golberg, 1970). Conversion is brought about mainly by the intestinal microflora in man (Drasar, Renwick & Williams, 1972; Golberg *et al.* 1969) and animals (A. J. Collings, unpublished data 1971; Drasar *et al.* 1972; Golberg *et al.* 1969; Parekh *et al.* 1970; Renwick & Williams, 1969).

Little is known of the metabolic fate of cyclohexylamine itself. Using ^{14}C-labelled material. Sonders, Estep & Wiegand (1968) showed that, in rats and dogs, it is readily distributed throughout the body tissues. Activity was more easily demonstrable in lung, spleen, liver and intestine than in other tissues. Most of the activity was excreted in urine within 24 hr and 11–27% of the administered material was in the form of metabolites. In man only 2% of the dose was presented as metabolites. Renwick & Williams (1972a) carried out similar studies in rats, guinea-pigs, rabbits and man. In the rat, guinea-pig and man at least 90% of an oral dose of CHA was excreted unchanged, mainly in the urine, whereas in the rabbit approximately 30% was metabolized. In the rat, four different aminocyclohexanols were identified, indicating that the metabolism was mainly by ring hydroxylation, although traces of cyclohexanol were also found. On the other hand, in man the metabolism appears to be mainly by deamination, producing cyclohexanol and cyclohexane-1,2-diol. Eight metabolites were found in the rabbit and six in the guinea-pig, and these suggest that ring hydroxylation and deamination occur in these species.

The LD_{50} of CHA in mice after ip injection was 619 mg/kg (Miyata, Kase, Kamikawa, Kataoka, Kikuchi & Touchi, 1969), while Pliss (1958) found the sc LD_{50} of an oil solution to be 1150 mg/kg. An injection of 500 mg/kg killed rabbits, while ten daily doses of 250 mg/kg were without effect (F. B. Flinn, unpublished data cited from Carswell & Morrill, 1937). In rats, an oral dose of 500 mg/kg caused death with signs of laboured respiration but doses of up to 150 mg/kg were not lethal. Lomonova (1965) found that the lethal concentration of CHA in the air was 4·3 and 11·5 mg/litre for mice and rats respectively. The signs of intoxication were increased excitability, weight loss, hypothermia, oliguria, methaemoglobinaemia and reticulocytosis. The weights of lung, heart, kidney and adrenals were increased and oedema of the brain and lung and degenerative changes of the heart and kidney were said to occur. Increased haemosiderin deposition was seen in the spleen, liver and lung.

No adequate short-term studies have been reported. F. B. Flinn (unpublished data cited from Carswell & Morrill, 1937) gave daily oral doses of 100 mg/kg to unspecified numbers of rabbits, rats and guinea-pigs for 82 days. No controls are mentioned but the treated animals gained weight and no abnormalities were seen at autopsy. Lomonova (1965) also exposed rats to the vapour of CHA at either 0·7 mg/litre for 2 hr each day for 2 months or at 0·1 mg/litre for 4 hr each day for 5 months. At the higher concentration, 50% of the animals died but only 5% died at the lower concentration. At both exposure levels, hypothermia, reductions in respiratory rate and oxygen consumption, an increase in kidney weight and diarrhoea were observed. At the higher concentration, the rate of body-weight gain was reduced and the heart weight increased. Histologically, inflammation of the respiratory system, degenerative changes of the heart and kidney and haemosiderin deposition in the spleen, liver and lung were seen. The histological appearance suggested that the activity of the thyroid was reduced.

Similarly, there is very little evidence on the effects of CHA in animals following prolonged exposure. In rats given 25 mg CHA/day in their food for 6 days/wk for 1 yr, no tumours were found in the 28 animals that survived for longer than 2 months (Pliss, 1958).

Price, Biava, Oser, Vogin, Steinfeld & Ley (1970) fed CHA sulphate at dietary levels equivalent to 0·0, 0·15, 1·5 and 15 mg/kg/day to groups of 25 rats of each sex for 2 yr. A high mortality in all groups was not related to dosage. Weight gain was reduced in male rats at the highest dosage level but no effects were detected on haemopoiesis, blood chemistry or the histological appearance of any organ except the bladder. In the bladder of one male from the highest treatment level there was a non-metastasizing, invasive transitional-cell carcinoma. The same workers fed a 10:1 cyclamate–saccharin mixture to rats at dietary levels equivalent to intakes of 0, 500, 1120 and 2500 mg/kg/day for 2 yr. Some of the rats converted cyclamate to CHA and, in addition, CHA was added to the diet of half the rats to give intakes of 25, 56 and 125 mg/kg/day from wk 79 of the study. Eight bladder tumours occurred in rats on the highest intake of the cyclamate–saccharin mixture; seven of these rats had been shown to convert cyclamate to CHA. The occurrence of tumours did not appear to be related to the addition of CHA to the diet, as five of the tumours occurred in rats not given the CHA supplement.

The present study was initiated as part of the BIBRA safety evaluation programme to assess the effects of CHA fed over a short term to rats and was intended as a preliminary to long-term studies in rats and mice, studies which are in progress at present. The lowest dose level of CHA hydrochloride used in the present study (approximately 30 mg/kg/day) was calculated to be equivalent to the dose of CHA that might be produced by an individual consuming 50 mg cyclamate/kg/day and converting all of the dose to CHA. The level of 50 mg cyclamate/kg/day is the maximum daily intake suggested in the Eleventh Report of the Joint FAO/WHO Expert Committee on Food Additives (1968).

EXPERIMENTAL

Materials. The CHA hydrochloride used in these studies was prepared by Laporte Industries, Ilford, Essex, from CHA conforming to the specification of the British Standards Institution (1968).

Animals and diet. Both sexes of the CFE strain of rat obtained from an SPF colony were housed in an animal room maintained at 21 ± 1°C with a relative humidity of 50–60%. They were fed Spillers' Laboratory Small Animal Diet and given water *ad lib.*, except where otherwise specified.

Experimental design and conduct

Acceptability of test diets. Pairs of male rats (200–250 g body weight) were housed in cages with access to both control diet and a second diet containing either 500, 1000, 3000 or 6000 ppm CHA. The amount of each diet consumed was recorded daily for 8 days. Pairs of male rats (370–400 g body weight) were fed a diet containing either 0 (control), 4000 or 6000 ppm CHA for 13 days with daily measurement of body weight and food intake.

Short-term feeding study. Groups of fifteen male rats (80–130 g body weight) and fifteen females (70–110 g body weight) were fed diets containing 0 (control), 600, 2000 or 6000 ppm CHA for 13 wk. Additional groups of five rats of each sex were fed diets containing 0, 2000 or 6000 ppm CHA for 3 or 6 wk. Body weight, food intake and water intake were measured weekly. After the appropriate feeding period the rats were deprived of food overnight and killed by exsanguination under barbiturate anaesthesia. Blood samples were collected from the aorta for haematological examination and serum analyses.

An autopsy was carried out, during which any macroscopic abnormalities were noted and the brain, pituitary, thyroid, heart, liver, kidney, adrenal glands, spleen, gonads, sto-

mach, small intestine and caecum were weighed. Samples of these organs and of trachea, lung, lymph nodes, thymus, urinary bladder, colon, rectum, pancreas, uterus and skeletal muscles were preserved in 10% buffered formalin. Paraffin-wax sections of the tissues from control rats and rats fed 6000 ppm CHA were stained with haematoxylin and eosin for histological examination. At the lower levels only the testes were examined histologically.

The haemoglobin concentration and packed cell volume were measured and counts were made of total erythrocytes, reticulocytes, total leucocytes and the various types of leucocytes. Serum was analysed for urea, glucose, total protein and albumin and for the activities of glutamic–pyruvic transaminase, glutamic–oxalacetic transaminase and lactic dehydrogenase.

Urine collected during the last week of feeding was examined for cells and other microscopic constituents and for content of glucose, ketones, bile and blood. A concentration test was carried out by measuring the volume and specific gravity of urine produced during a 6-hr period of water deprivation. At wk 6 and 13 this study was extended by making the same measurements on the urine produced in a 2-hr period after a water load of 25 ml/kg and on urine produced during a 4-hr period commencing 16 hr after the water load.

Paired feeding study. Groups of five rats of each sex were housed individually and fed on diets containing either 0 (control) or 6000 ppm CHA for 90 days. Each control rat was from the same litter as one of the treated rats. The treated rats were fed *ad lib.* and the food intake of each was measured daily. Each control was fed an amount equal to that consumed in the previous 24 hr by its litter-mate partner. Body weight was measured weekly.

Studies of intestinal absorption of nutrients. Groups of ten male rats (body weight 90–120 g) were fed diets containing 0 (control) or 6000 ppm CHA for 26 wk and nutrient absorption was determined as wk 13 and 26. Carbohydrate absorption was assessed by estimating the 6-hr urinary excretion of xylose (Varley, 1967) following administration of an oral dose of 200 mg xylose as an aqueous solution in a volume of 25 ml/kg. Fatty acid and protein absorption were determined in rats housed individually in metabolism cages and fed on a diet containing approximately 34% protein and 20% fat. This diet consisted of 61% Spillers' Laboratory Small Animal Diet, 22% casein and 11% corn oil, and for the treated animals CHA was incorporated at 6000 ppm. The rats were fed on the diet for 8 days followed by 24 hr without food. The food intake was measured over the last 4 days of feeding and faeces were collected for the last 3 days of feeding and during the subsequent 24-hr fast. The protein and fatty acid contents of the diet and faeces were estimated (Varley, 1967) and the percentage absorption was calculated.

Measurement of basal metabolic rate. Studies of oxygen consumption, using an apparatus based on the principle described by Capraro (1953), were made on the animals previously used for the intestinal absorption studies after the test feeding had been continued for 10 months and on two groups of nine rats pair-fed for 4 wk with diet containing 0 or 6000 ppm CHA. Measurements were made on one treated and one control rat each day and the order in which the treatments were studied was changed. In the case of the pair-fed animals the litter-mate partners were studied on the same day. Each rat was allowed 60–90 min to settle in the apparatus, and after this readings were taken at approximately 30 min intervals for 2·5–3 hr. The lowest reading of oxygen consumption obtained was taken to be the basal rate.

Reproduction study. Groups of five male rats fed either 0 or 6000 ppm CHA for 10 months were used. Each male was caged for 10 days with three young untreated females.

Treatment of the males was continued through the mating period. Records were kept of the number of young born and the sex ratio, survival rate and weight gain. Offspring that died and all survivors at day 21 were examined for macroscopic abnormalities.

RESULTS

Acceptability tests

Rats offered untreated and treated food ate almost no diet containing 3000 or 6000 ppm CHA, but at the lower levels (500 and 1000 ppm CHA) some of the treated diet was consumed. This applied not only during the first days of feeding but throughout the 8-day test period, over which the daily intake of the treated diets (with the intake of untreated diet in parentheses) was 4·8 (9·4) and 4·1 (10·6) g/rat for the 500 and 1000 ppm diets compared with 0·3 (15·8) and 0·1 (12·9) g/rat for the 3000 and 6000 ppm diets, respectively. When no choice of diet was given, the rats consumed the food containing 4000 or 6000 ppm CHA, but intake was lower than with the basic diet, the total 13-day intakes of these three diets being 220, 194 and 282 g/rat, respectively. However, comparison of the amount eaten in the first 3 and the last 3 days showed that the rejection of the diet was more marked at the beginning than at the end of the study.

Short-term feeding study

No abnormalities were seen in the condition or behaviour of any of the rats but at the two highest dosage levels (2000 and 6000 ppm) the rats were noticeably smaller than the controls. This was reflected in the rate of body-weight gain, which was significantly reduced in both sexes fed a diet containing 2000 or 6000 ppm CHA (Table 1). This reduction of weight gain was evident from day 1; over the first 24 hr the control rats gained approximately 7 g, whereas rats fed 2000 ppm CHA gained only 2 g and at the highest dietary level there was a 1 g loss in body weight. At the 600 ppm level body-weight gain was unaffected. The food intake was somewhat reduced at all dietary levels (Table 1), the most marked reduction being in the first 24 hr. The mean intake over the whole experimental period was lowered to a statistically significant degree in both sexes fed diets containing 2000 or 6000 ppm CHA and at the 600 ppm level in females. The water consumption (Table 1) was slightly reduced at all dietary levels; the differences in mean intake over the whole period of study were statistically significant in all rats fed a dietary level of 6000 ppm CHA and in females given the 2000 ppm dietary level. The intakes of CHA calculated from body-weight and food-consumption data averaged out over the study to 41, 143 and 468 mg/kg/day for the 600, 2000 and 6000 ppm diets respectively.

There were no differences between treated and control animals in the haematological findings at wk 3. At wk 6 the total leucocyte count was lower in females fed 2000 ppm CHA than in controls, but this difference was not seen at the higher level or in males. At wk 13 the total white count was reduced in males fed 2000 or 6000 ppm CHA (Table 2). No similar effect was seen in females and the decrease was not associated with any particular type of leucocyte. The haemoglobin and packed cell volume were reduced in males fed the diet containing 6000 ppm CHA for 13 wk. In addition, the packed cell volume was reduced in the males given 2000 ppm CHA. Conversely the haemoglobin and red cell count were increased in females fed 6000 ppm CHA.

There were no differences between treated and control animals in the results of serum analyses for glucose, urea, proteins, transaminases and lactic dehydrogenase. The number

Table 1. *Mean body weight and food and water consumption of rats fed CHA at 0–6000 ppm of the diet for 90 days*

Dietary level (ppm)	Body weight (g) at day				Weight gain (g) to day 84	Food consumption (g/rat/day) at day				Mean consumption (g/rat/day) to day 84	Water consumption (g/rat/day) at day				Mean water consumption (g/rat/day) up to day 84
	0†	25	56	84		1	25	56	84		7	25	56	84	
								Males							
0	99	291	417	471	372	15·0	23·7	21·9	21·7	21·4	24·7	31·9	29·9	24·9	27·8
600	97	286	411	474	377	13·5	22·1	20·2	20·9	20·5	23·8	25·9	28·8	25·4	25·4
2000	97	264**	380**	435**	338**	11·9	22·3	20·3	20·8	20·0*	22·5	24·0	26·1	24·4	25·2
6000	97	231***	328***	371***	274***	9·1	20·9	19·0	18·7	18·6***	18·2	19·8	21·5	19·7	19·3***
								Females							
0	80	192	249	281	201	12·7	18·0	16·1	16·7	16·5	21·4	20·2	23·5	21·3	21·7
600	81	186	244	271	190	11·7	15·8	15·3	15·8	14·9**	19·5	20·4	26·1	23·0	21·8
2000	82	168***	219***	242***	160***	8·7	14·0	14·0	14·8	14·3***	17·7	18·2	22·7	21·7	19·5**
6000	81	154***	197***	214***	133***	6·7	15·7	13·8	14·5	13·9***	16·9	16·2	19·3	22·3	17·8**

† First day of feeding.

Body weights are means for groups of 15 animals, and food and water consumption figures are means for three cages of five animals. Those marked with asterisks differ significantly (Student's t test for body weights and Ranking method of White (1952) for mean food and water consumption) from those of controls: $*P < 0·05$; $**P < 0·01$; $***P < 0·001$.

Table 2. *Haematological findings in rats fed CHA at 0–6000 ppm of the diet for 13 wk*

Dietary level (ppm)	Hb (g/100 ml)	PCV (%)	RBC (10^6/mm^3)	Retics (% of RBC)	Leucocytes: Total (10^3/mm^3)	Differential (%): N	E	L	M
Males									
0	15·3	48	8·41	0·7	8·31	22	2	74	2
600	14·8	47	7·97	0·8	6·68	17	2	78	3
2000	14·9	45*	8·03	0·9	6·36*	18	2	78	2
6000	14·7*	46*	8·02	0·8	6·27*	18	2	78	2
Females									
0	13·6	43	6·60	1·1	4·40	17	2	80	1
600	13·7	44	7·05	1·2	4·23	16	1	81	2
2000	13·9	45	7·22	0·9	4·84	17	1	80	2
6000	14·2*	44	7·37*	1·2	4·39	15	2	82	1

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

Figures are means for groups of 15 rats and those marked with an asterisk differ significantly (Student's *t* test) from those of controls: *$P < 0·05$.

Basophils were not encountered at levels of 1% or more of the leucocytes and no inclusions were seen in the erythrocytes.

of cells excreted in the urine was similar in control and treated rats. At wk 3 and 6 there were no significant differences between treated and control animals in the results of the renal concentration test. However, females fed for 13 wk on a diet containing 2000 or 6000 ppm CHA produced urine that was slightly less concentrated after a 16-hr water deprivation than that from the female controls ($P < 0·05$). No significant effects were seen in males.

At autopsy no gross abnormalities were seen in rats fed on diets containing 600 or 2000 ppm CHA. At the highest level (6000 ppm), no abnormalities were seen at wk 3 or 6 but at wk 13 the testes were visibly reduced in size. No differences from controls were seen in the organ weights of rats fed 6000 ppm CHA but at the higher levels the weights of most organs were reduced (Table 3). The body weights also were decreased and most of the differences in organ weight were eliminated when the weights were expressed relative to body weight (Table 4). In the relative weights the only consistent changes were increases in brain weight in the rats given 2000 or 6000 ppm and increases in stomach weight at 6000 ppm. The relative weights of the testes of the rats fed the diet containing 6000 ppm CHA were increased at wk 3 and 6 but decreased at wk 13. The relative ovary weights were increased at all three intervals. In addition, isolated increases in relative organ weights were found in the kidney, caecum, small intestine. adrenal glands and thyroid. Some decreases were found in the heart and liver.

Histological examination showed slight fatty infiltration in the livers of the females and some alveolar thickening and lymphocyte infiltration in the lungs of both sexes. These changes were similar in incidence and severity in treated and control animals. The only histological finding that could be related to treatment was reduced spermatogenesis and tubular atrophy in the testes. This was not seen in rats fed on diet containing 600 ppm CHA, whereas four of the 11 animals from the 2000 ppm CHA group and 18 of 20 fed on the diet containing 6000 ppm were affected. The severity of the effect was greater in

Table 3. *Organ weights of rats given CHA at 0–6000 ppm of the diet for 3, 6 or 13 wk*

Sex and dietary level (ppm)	No. of rats	Organ weight (g) Brain	Heart	Liver	Spleen	Kidneys	Stomach	Small intestine	Caecum	Adrenals†	Gonads‡	Pituitary†	Thyroid†	Body weight (g)
								Wk 3						
Male														
0	5	1·75	0·86	7·47	0·75	1·88	1·25	6·78	0·75	58·7	2·23	8·9	14·0	208
2000	5	1·76	0·81	6·90	0·76	1·75	1·17	5·99*	0·79	48·7*	2·43	7·8	17·1	204
6000	5	1·65*	0·60***	5·51***	0·55***	1·52*	1·19	5·89*	0·65*	47·4*	2·49	6·8	12·5	157**
Female														
0	5	1·64	0·78	5·80	0·66	1·51	1·05	5·83	0·60	65·0	127	8·9	14·7	161
2000	5	1·62	0·60***	4·96*	0·54*	1·31**	0·93**	5·05*	0·58	74·0	152	8·3	12·2*	140*
6000	5	1·65	0·59***	4·69**	0·53**	1·24***	0·93**	4·61**	0·56	63·6	148	7·0	11·5*	128**
								Wk 6						
Male														
0	5	1·84	1·09	9·94	0·85	2·43	1·47	7·21	0·84	65·1	3·74	10·8	17·1	317
2000	5	1·92	1·04	7·96***	0·77	2·20	1·33	6·13*	0·83	49·9*	3·48	10·6	17·4	290
6000	5	1·85	0·92*	8·31***	0·66**	2·11*	1·37	6·23*	0·75	56·8	3·69	8·8*	20·4	279*
Female														
0	5	1·67	0·80	6·31	0·67	1·60	1·20	5·95	0·67	82·4	126	11·5	14·1	214
2000	5	1·70	0·67**	5·41*	0·57*	1·50	1·12	5·05*	0·59	63·5**	116	10·8	14·2	182**
6000	5	1·60	0·65**	4·85**	0·49**	1·30**	1·08	4·56**	0·58*	55·2***	116	8·6**	11·9	168**
								Wk 13						
Male														
0	15	1·95	1·33	12·67	0·81	3·19	1·77	7·78	1·04	66·8	3·74	12·4	23·6	466
600	15	1·91	1·29	12·76	0·82	3·30	1·79	7·99	0·99	64·6	3·70	12·4	24·1	465
2000	15	1·87*	1·22**	10·93*	0·75	2·83**	1·66	7·34	0·89	61·9	3·43	10·7	21·3	427*
6000	15	1·81*	1·08**	10·44**	0·63***	2·49***	1·65*	6·59***	0·77***	57·1	2·43***	10·2	19·9**	365***
Female														
0	15	1·74	0·88	6·49	0·58	1·79	1·38	6·26	0·70	66·1	122	13·4	18·3	284
600	15	1·74	0·87	6·79	0·58	1·79	1·35	6·30	0·72	67·5	125	13·8	19·1	272
2000	15	1·72	0·73**	5·63***	0·53**	1·63**	1·27	5·31***	0·65	62·3	135	11·7*	17·5	244***
6000	15	1·69	0·64***	4·85***	0·45***	1·37***	1·25	5·08***	0·64	55·5***	116	10·3***	15·6	211***

† Weights of these organs are expressed in mg.
‡ Weights of female gonads are expressed in mg.
Figures are means for the numbers of rats shown and those marked with asterisks differ significantly (Student's t test) from those of controls: $*P < 0{\cdot}05$; $**P < 0{\cdot}01$; $***P < 0{\cdot}001$.

Table 4. *Relative organ weights of rats fed diets containing CHA at 0–6000 ppm for 3, 6 or 13 wk*

Sex and dietary level (ppm)	No. of rats examined	Relative organ weight (g/100 g body weight)											
		Brain	Heart	Liver	Spleen	Kidneys	Stomach	Small intestine	Caecum	Adrenals†	Gonads‡	Pituitary†	Thyroid†
							Wk 3						
Male													
0	5	0·85	0·41	3·61	0·36	0·91	0·60	3·29	0·36	28·1	1·07	4·5	7·0
2000	5	0·86	0·39	3·38	0·37	0·85	0·57	2·97	0·39	24·5	1·19	3·8	8·4
6000	5	1·07*	0·38	3·51	0·35	0·97	0·77*	3·80	0·42	30·3	1·56***	4·3	8·0
Female													
0	5	1·03	0·48	3·62	0·41	0·95	0·66	4·22	0·37	40·5	80	5·4	9·2
2000	5	1·16	0·43*	3·55	0·37	0·93	0·67	3·61	0·42	53·2	110	5·9	8·7
6000	5	1·29*	0·46	3·65	0·41	0·96	0·73	3·50	0·44	49·5	115*	5·5	8·9
							Wk 6						
Male													
0	5	0·58	0·34	3·14	0·27	0·76	0·46	2·27	0·27	20·5	1·10	3·4	5·4
2000	5	0·66**	0·32	2·73**	0·26	0·75	0·46	2·10	0·28	17·1	1·19	3·6	5·9
6000	5	0·66***	0·35	2·98	0·23	0·75	0·49	2·23	0·26	20·4	1·32***	3·1	7·3
Female													
0	5	0·78	0·37	2·94	0·31	0·75	0·56	2·77	0·31	38·6	59	5·4	6·6
2000	5	0·94**	0·36	2·90	0·31	0·85***	0·62	2·77	0·32	34·2	64	6·0	7·8
6000	5	0·96**	0·38	2·88	0·29	0·77	0·64**	2·71	0·35	33·0*	69	5·1	7·1
							Wk 13						
Male													
0	15	0·42	0·29	2·72	0·18	0·69	0·38	1·67	0·23	14·4	0·81	2·7	5·1
600	15	0·41	0·28	2·74	0·18	0·71	0·39	1·72	0·21	14·0	0·80	2·7	5·2
2000	15	0·44	0·29	2·56	0·18	0·66	0·39	1·71	0·21	14·5	0·81	2·5	5·1
6000	15	0·50***	0·29	2·79	0·17	0·68	0·45***	1·81*	0·21	15·8	0·67*	2·8	5·1
Female													
0	15	0·62	0·31	2·29	0·21	0·63	0·49	2·21	0·25	23·4	43	4·7	6·1
600	15	0·63	0·32	2·30	0·21	0·66	0·49	2·31	0·26	24·8	45	5·1	7·1
2000	15	0·70***	0·30	2·30	0·22	0·67	0·52	2·16	0·27	25·5*	56**	4·8	7·2
6000	15	0·80***	0·30	2·29	0·22	0·65	0·59**	2·40	0·31*	26·5*	55**	4·9	7·4*

† Relative weights of these organs are expressed as mg/100 g body weight.
‡ Relative weights of female gonads are expressed as mg/100 g body weight.
Figures are means for the numbers of observations shown and those marked with asterisks differ significantly (Student's *t* test) from those of controls: $^{*}P < 0{\cdot}05$; $^{**}P < 0{\cdot}01$; $^{***}P < 0{\cdot}001$.

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animals fed the highest concentration, eight rats from this group but none from the other showing complete arrest of spermatogenesis and loss of germinal epithelium.

Paired-feeding study

The body-weight gain of rats fed the diet containing 6000 ppm CHA was lower than that of the pair-fed controls despite the similarity of food intakes (Table 5). As in the short-term study, a loss of weight occurred in the treated animals during the first day but the difference between treated and control animals did not reach statistical significance until wk 5, whereas in the short-term study, with *ad lib.* fed controls, the differences in body weight were significant by day 7.

After treatment for 13 wk the animals were killed and examined as in the short-term toxicity test. The macroscopic and histopathological findings were similar to those in the latter study. One male rat receiving 6000 ppm CHA had a grossly enlarged spleen containing numerous white nodules, which proved on histopathological examination to be due to a lymphosarcoma. This animal also had marked hyperplasia of the lymph nodes, an increase in the size of the Peyer's patches in the small intestine and infiltrations of lymphocytes and macrophages in the lungs and liver. No similar effect was encountered in any of the other rats treated with CHA in the present series of experiments.

The organs of the treated rats weighed less than those of the controls (Table 6) but the differences were less marked than in the short-term study. When the organ weights were expressed relative to body weight the testis weight was again reduced but no increase in ovary weight was found. As in the short-term study, the relative brain weight of the treated rats was significantly greater than that of the controls in both sexes and the relative thyroid weight was increased in males.

Intestinal absorption studies

The absorption of carbohydrate, fatty acids and protein was the same in rats fed on a diet containing 6000 ppm CHA for 3 or 6 months as in similarly aged controls. At 3 months the mean values for urinary excretion of xylose, fatty acid absorption and protein absorption were 23, 97 and 91% in the test group, compared with 26, 97 and 90% in controls, while at 6 months the corresponding test and control values were 34, 97 and 90% and 28, 97 and 89%, respectively.

Table 5. *Body weight of rats fed CHA at 6000 ppm of the diet and of pair fed controls*

Dietary level (ppm)	Body weight (g) at day						Weight gain (g)	Total food consumed (g)
	0†	1	18	42	70	91		
				Male				
0	89	94	190	305	373	401	312	1336
6000	90	89	177	266**	334**	357**	267*	1325
				Female				
0	94	97	154	212	250	265	171	1513
6000	91	88	138	188*	213**	218**	127**	1541

† Day 1 of feeding.

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

Table 6. *Organ weights and relative organ weights of rats fed 6000 ppm CHA for 13 wk compared with those of pair-fed controls*

Sex and dietary level (ppm)	Organ weights												Body weight (g)
	Brain	Heart	Liver	Spleen	Kidneys	Stomach	Small intestine	Caecum	Adrenals†	Gonads‡	Pituitary†	Thyroid†	
							Organ weight (g)						
Male													
0	1·89	1·28	11·94	0·78	2·93	1·82	8·36	0·94	65	3·58	11·9	21·1	416
6000	1·79	1·10*	9·39*	0·61*	2·43*	1·54	8·08	0·94	53	1·54*	13·6	22·7	354**
Female													
0	1·67	0·88	6·85	0·55	1·77	1·41	7·12	0·61	80	166	12·0	15·4	268
6000	1·63	0·70**	5·87	0·40**	1·57*	1·24	6·47	0·67	60**	121	9·7	13·6	224**
							Relative organ weight (g/100 g body weight)						
Male													
0	0·45	0·31	2·87	0·19	0·70	0·44	2·01	0·23	16	0·86	2·9	5·1	
6000	0·50*	0·31	2·65	0·17	0·69	0·43	2·28	0·27	15	0·44**	3·8	6·4*	
Female													
0	0·63	0·33	2·56	0·20	0·66	0·53	2·66	0·23	30	62	4·5	5·8	
6000	0·73*	0·31	2·62	0·18	0·70	0·55	2·89	0·30*	27	54	4·3	6·1	

† The weights and relative weights for these organs and the female gonads are expressed in mg and mg/100 g body weight respectively.
‡ The weights and relative weights for the female gonads are expressed in mg and mg/100 g body weight.
The figures are means for groups of four male rats and five females and those marked with asterisks differ significantly (White, 1952) from the control values: $*P < 0{\cdot}05$; $**P < 0{\cdot}01$.

Studies of basal metabolic rate

Rats fed for 10 months on diet containing 6000 ppm CHA utilized less oxygen in total than similarly aged controls fed *ad lib.*, but on the basis of oxygen consumed/100 g body weight, six of the ten treated rats showed an increased oxygen consumption (Table 7). When the comparison was made using pair-fed controls the oxygen utilization of the CHA-treated rats was increased by approximately 10% both in terms of total oxygen and in relation to body weight, although CHA treatment had continued only for 4 wk.

Table 7. *Oxygen consumption of rats fed on diets containing 0 or 6000 ppm CHA*

Treatment	No. of estimations	Oxygen consumption ml/hr/rat	Oxygen consumption ml/hr/100 g body weight	No. of cases in which test exceeded control
		Month 10		
Ad lib. controls	10	338	55·8	
6000 ppm CHA	10	317	60·9	6/10
		Wk 4		
Pair-fed control	9	216	99·3	
6000 ppm CHA	9	246	110·4	9/9

Figures are means for the numbers of estimations shown. One treated and one control rat were studied on each day.

Reproduction study

There were no statistically significant differences between treated and control groups in the number of fertile males or in litter size and growth (Table 8). There were, on average, more females in the litters sired by treated males but this was due mainly to one litter consisting of two males and 11 females. Apart from this, control and test animals were similar.

Table 8. *Results of fertility study using untreated females and males treated with 0 or 6000 ppm CHA for 10 months*

Dietary level (ppm)	Day	Mean no. of pups	Sex ratio (male/female)	Total weight of litter (g)	Mean pup weight (g)
0	0*	13·5	1·08	71·3	5·9
	4	11·2	1·15	92·8	8·1
	14	10·3	1·24	290·3	27·0
	21	10·3	1·24	462·3	43·7
6000	0*	9·7	0·54	70·5	7·5
	4	9·7	0·54	108·8	11·6
	14	9·7	0·54	329·0	35·5
	21	9·7	0·54	518·7	55·8

*Data at birth.
Test and control data were derived from 15 females divided into groups of three, each group being housed for 15 days with one male. Three out of the five males proved fertile in both the test and control groups, in each of which there were seven pregnant females. One litter from each group was cannibalized at birth so the data given above are in both groups derived from six litters.

DISCUSSION

In this study no adverse effects were detected in rats fed CHA hydrochloride at 600 ppm in the diet for 90 days. At higher dietary levels the rate of body-weight gain was reduced

and this was accompanied by a reduction in the food intake. The facts that diets containing CHA were avoided by rats when control diet was available and that the food intake was reduced, particularly in the first days of feeding, when only diet containing CHA was available suggest that diet containing CHA was unpalatable. The reduced food intake does not entirely account for the reduced weight gain since treated animals also showed a reduction in the rate of weight gain compared with pair-fed controls. The absorption studies show that the failure to gain weight is unlikely to be attributable to impaired gastro-intestinal absorption of nutrients. The total oxygen consumption under basal conditions by rats fed CHA for long periods was not consistently different from that of controls. However, these data were not strictly comparable, since the body weights of test and control rats were different (controls 660 g, test rats 510 g). In the paired-feeding experiment, in which the body weights of control and test rats were similar, the basal oxygen consumption by the rats fed a diet containing CHA was approximately 10% greater than that of the controls. The difference (30 ml/rat/hr or 700 ml/day) is equivalent to approximately 3·4 Kcal/day. Kaunitz, Slanetz & Johnson (1957) found that approximately 1 g of food (equivalent to 3 Kcal) was needed to increase the body weight of rats by 1 g and this was confirmed by Sharratt (1961). Thus the difference in basal metabolic rates could account entirely for the reduction of approximately 0·55 g/day in the rate of gain in body weight of the CHA-treated rats.

An increased oxygen utilization is in keeping with the observation of Lee & Dixon (1969) that there was an increased production of carbon dioxide from ^{14}C-labelled acetate in animals treated with CHA. The mechanism by which CHA increases the basal metabolic rate is not known. It has been shown (Rosenblum & Rosenblum, 1968a, b; Yamamura, Lee & Dixon, 1968) that CHA has a marked sympathomimetic effect on cardiac and other muscle and it is possible that this action might lead to a greater oxygen demand by the animal. However no obvious increase in activity was observed in the animals in the present study. It is also possible that the effect may be mediated by way of the thyroid, as the relative thyroid weights were increased when compared with *ad lib.* controls or pair-fed controls.

The changes in total leucocyte count found at wk 6 and 13 were not seen in both sexes and at wk 6 were not seen at the highest dose level. In addition, it was noticed that no particular type of leucocyte was affected; thus this effect seems unlikely to be related to treatment with CHA. The anaemia in the males fed for 13 wk on diets containing 2000 or 6000 ppm CHA was also unlikely to have been due to CHA treatment, as increases were seen at the same dose levels in the females. It was thought possible that it might be due to gastro-intestinal haemorrhage consequent upon irritation caused by CHA. However, this does not seem to have been the case, since no anaemia was seen in females, occult blood was absent from the faeces of five male rats fed 6000 ppm CHA for 21 months (I. F. Gaunt, unpublished observation 1972), there was no histological evidence of irritation of the gastro-intestinal tract and there was no reticulocytosis. The acute inhalation studies of Lomonova (1965) showing a methaemoglobinaemia, haemosiderosis of the spleen, lung and liver and a reticulocytosis, suggest an increased red cell turnover associated with an increased destruction of erythrocytes. In the present study, splenic haemosiderosis was not seen and the number of reticulocytes in the blood was not increased, so the anaemia could not be attributed to an increased red-cell destruction.

The majority of the changes in organ weight are consistent with failure of the rats to gain weight at the normal rate. Similar increases in the relative weight of brain, stomach,

small intestine and adrenals have been found in rats with dietary intakes of control food restricted to 90% of their normal intake (M. Sharratt, unpublished data 1970).

The testis weight and the weight of the testes relative to body weight were notably decreased in the rats fed 6000 ppm CHA for 13 wk. The testes were visibly smaller at autopsy and, on micropathological examination, marked atrophy of the testes was seen at this level of treatment. It also occurred at the 2000 ppm dietary level. However, despite this atrophy and the marked reduction of spermatogenesis, the reproduction test showed that the rats remained fertile. This finding of normal fertility was based on results from a small number of animals and so can be regarded only as a preliminary observation, but it is in agreement with the work of Kennedy, Sanders, Weinberg, Arnold & Keplinger (1969), who found no adverse effects on reproduction in rats and rabbits given daily doses of 1·5 or 15 mg CHA/kg. In their study a slight impairment of male fertility was found in rats given higher dosage levels, although this effect did not appear to persist into the second and third generation.

An important feature of the fertility study was the absence of any teratological effect. This was in keeping with the failure of Cattanach & Pollard (1971) to detect any effect in a dominant lethal test in mice. However, CHA has been shown to produce chromosomal damage in rats. Thus Legator, Palmer, Green & Petersen (1969) found a dose-related increase in the number of chromosome breaks in spermatogonial and bone-marrow cells 24 hr after 5 daily ip doses of 1–50 mg/kg. The effect was confined to the germinal cells at the lowest dose level. However, Cattanach & Pollard (1971) found no effect in mice treated in a similar manner.

The mechanism by which testes are affected is not yet known. It was thought possible that the effect of CHA in increasing the tone of the muscle of the vas deferens (Rosenblum & Rosenblum, 1968b) might be responsible. However, this seems unlikely as unilateral ligation of the vas deferens for periods of up to 4 months does not affect the testis weight (I. F. Gaunt, unpublished information 1971).

The lack of any effect on the development of the young of parents treated with CHA is in keeping with the findings of Kennedy *et al.* (1969) and Khera, Stoltz, Gunner, Lyon & Grice (1971), despite the fact that CHA has been shown, at least in primates, to cross the placenta (Pitkin, Reynolds & Filer, 1969).

The slight reduction in the concentration of urine passed after a 16-hr period of water deprivation might suggest that the 6000 ppm CHA diet had caused some renal damage. However, this alteration in function was not accompanied by other indications of kidney damage and the finding is of doubtful toxicological significance.

No changes in bladder epithelium were found in rats receiving CHA. The lymphosarcoma found in one rat on the 6000 ppm diet could not be attributed to CHA treatment in the absence of any similar findings in the remainder of the animals and the common occurrence of the lesion in the strain of rats used.

The no-untoward-effect level established for CHA in this study was 600 ppm of the diet, a level approximately equal to 30 mg/kg/day. Use of a 100-fold safety factor would give a calculated acceptable daily intake (ADI) of 0·3 mg CHA/kg/day, or approximately 20 mg/day for a 70-kg adult and 9 mg/day for a 30-kg child. If all cyclamate is converted to CHA, this is equivalent to an ADI for cyclamate of 40 mg/day in an adult. The highest reported conversion for cyclamate to CHA is 60% (A. J. Collings, unpublished data 1971), so that an ADI of 60–70 mg/day might be justified.

Toxicological investigations such as those reported here can only be considered as preliminary tests. However, the present work provides some guidelines for further studies to determine the mechanism of action of CHA, and in particular the effect on the testes and whether this mechanism is likely to occur in man at the levels of CHA liable to be produced by conversion of ingested cyclamate.

REFERENCES

Barger, G. & Dale, H. H. (1910). Chemical structure and sympathomimetic action of amines. *J. Physiol., Lond.* **41**, 19.

British Standards Institution (1968). Specification for Cyclohexylamine. BS 2534:1968.

Capraro, V. (1953). A new method of measuring oxygen consumed in the metabolism of small animals. *Nature, Lond.* **172**, 815.

Carswell, T. S. & Morrill, H. L. (1937). Cyclohexylamine and dicyclohexylamine. Properties and uses. *Ind. Engng Chem.* **29**, 1247.

Cattanach, B. M. & Pollard, C. E. (1971). Mutagenicity tests with cyclohexylamine in the mouse. *Mutation Res.* **12**, 472.

Drasar, B. S., Renwick, A. G. & Williams, R. T. (1972). The role of the gut flora in the metabolism of cyclamate. *Biochem. J.* **129**, 881.

Golberg, L., Parekh, C., Patti, A. & Soike, K. (1969). Cyclamate degradation in mammals and in vitro. *Toxic. appl. Pharmac.* **14**, 654.

Joint FAO/WHO Expert Committee on Food Additives—Eleventh Report (1968). Specifications for the Identity and Purity of Food Additives and their Toxicological Evaluation: Some Flavouring Substances and Non-Nutritive Sweetening Agents. *Tech. Rep. Ser. Wld Hlth Org.* **383.**

Kaunitz, H., Slanetz, C. A. & Johnson, R. E. (1957). Utilization of food for weight maintenance and growth. *J. Nutr.* **62**, 551.

Kennedy, G. L., Sanders, P. G., Weinberg, M. S., Arnold, D. W. & Keplinger, M. L. (1969). Reproduction studies in rats and rabbits with cyclohexylamine sulfate. *Toxic. appl. Pharmac.* **14**, 656.

Khera, K. S., Stoltz, D. R., Gunner, S. W., Lyon, D. A. & Grice, H. C. (1971). Reproduction study in rats orally treated with cyclohexylamine sulfate. *Toxic. appl. Pharmac.* **18**, 263.

Kojima, S. & Ichibagase, H. (1966). Studies on synthetic sweetening agents. VIII. Cyclohexylamine, a metabolite of sodium cyclamate. *Chem. pharm. Bull., Tokyo* **14**, 971.

Leahy, J. S., Taylor, T. & Rudd, C. J. (1967). Cyclohexylamine excretors among human volunteers given cyclamate. *Fd Cosmet. Toxicol.* **5**, 595.

Leahy, J. S., Wakefield, M. & Taylor, T. (1967). Urinary excretion of cyclohexylamine following oral administration of sodium cyclamate to man. *Fd Cosmet. Toxicol.* **5**, 447.

Lee, I. P. & Dixon, R. L. (1969). Factors affecting cyclohexylamine lethality in mice. *Toxic. appl. Pharmac.* **14**, 654.

Legator, M. S., Palmer, K. A., Green, S. & Petersen, K. W. (1969). Cytogenetic studies in rats of cyclohexylamine, a metabolite of cyclamate. *Science, N.Y.* **165**, 1139.

Lomonova, G. V. (1965). Toxicity of cyclohexylamine and dicyclohexylamine. *Fedn Proc. Transl. Suppl.* **24**, T96.

Miller, J. P., Crawford, L. E. M., Sonders, R. C. & Cardinal, E. V. (1966). Distribution and excretion of ^{14}C-cyclamate sodium in animals. *Biochem. biophys. Res. Commun.* **25**, 153.

Miyata, T., Kasé, Y., Kamikawa, Y., Kataoka, M., Kikuchi, K. & Touchi, T. (1969). Pharmacological characteristics of cyclohexylamine, one of the metabolites of cyclamate. *Life Sci.* **8**, 843.

Oser, B. L., Carson, S., Vogin, E. E. & Sonders, R. C. (1968). Conversion of cyclamate to cyclohexylamine in rats. *Nature, Lond.* **220**, 178.

Parekh, C., Goldberg, E. K. & Golberg, L. (1970). Fate of sodium cyclamate-^{14}C in the rhesus monkey (*M. mulatta*). *Toxic. appl. Pharmac.* **17**, 282.

Pitkin, R. M., Reynolds, W. Ann & Filer, L. J. (1969). Cyclamate and cyclohexylamine: Transfer across the hemochorial placenta. *Proc. Soc. exp. Biol. Med.* **132**, 993.

Pliss, G. B. (1958). The carcinogenic activity of dicyclohexylamine and its nitrite salt. *Probl. Oncol.* **4**, 659.

Price, J. M., Biava, C. G., Oser, B. L., Vogin, E. E., Steinfeld, J. & Ley, H. L. (1970). Bladder tumors in rats fed cyclohexylamine or high doses of a mixture of cyclamate and saccharin. *Science, N.Y.* **167**, 1131.

Renwick, A. G. & Williams, R. T. (1969). Gut bacteria and the metabolism of cyclamate in the rat. *Biochem. J.* **114**, 78P.

Renwick, A. G. & Williams, R. T. (1972a). The metabolites of cyclohexylamine in man and certain animals. *Biochem. J.* **129**, 857.

Renwick, A. G. & Williams, R. T. (1972b). The fate of cyclamate in man and other species. *Biochem. J.* **129**, 869.

Rosenblum, I. & Rosenblum, G. (1968a). Cardiovascular responses to cyclohexylamine. *Toxic. appl. Pharmac.* **12,** 260.

Rosenblum, I. & Rosenblum, G. (1968b). Autonomic stimulating and direct actions of cyclohexylamine in isolated tissues. *Toxic. appl. Pharmac.* **13,** 339.

Schoenberger, J. A., Rix, D. M., Sakamoto, A., Taylor, J. D. & Kark, R. M. (1953). Metabolic effects, toxicity and excretion of calcium N-cyclohexylsulfamate (sucaryl) in man. *Am. J. med. Sci.* **225,** 551.

Sharratt, M. (1961). The Assessment of Toxicity. Studies in Body Weight Changes. Ph.D. Thesis. University of Birmingham.

Sonders, R. C., Estep, C. B. & Wiegand, R. G. (1968). Metabolism of cyclohexylamine. *Fedn Proc. Fedn Am. Socs exp. Biol.* **27,** 238.

Taylor, J. D., Richards, R. K. & Davin, Jeanne C. (1951). Excretion and distribution of radioactive S^{35}-cyclamate sodium (sucaryl sodium) in animals. *Proc. Soc. exp. Biol. Med.* **78,** 530.

Varley, H. (1967). *Practical Clinical Biochemistry.* 4th ed. William Heinemann Medical Books Ltd., London.

White, C. (1952). The use of ranks in a test of significance for comparing two treatments. *Biometrics* **8,** 33.

Yamamura, H. I., Lee, I. P. & Dixon, R. L. (1968). Study of the sympathomimetic action of cyclohexylamine, a possible metabolite of cyclamate. *J. pharm. Sci.* **57,** 1132.

Fd Cosmet. Toxicol. Vol. 12 pp. 625–640. Pergamon Press 1974. Printed in Great Britain

ACCUMULATION OF INGESTED SULPHITE- AND SULPHATE-SULPHUR AND UTILIZATION OF SULPHITED PROTEINS BY RATS

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Abstract—The accumulation of ^{35}S in various tissues of male and female albino rats fed [^{35}S]sulphate or [^{35}S]sulphite in daily doses equivalent to 50 mg SO_2/kg for 2, 7 and 14 days was compared. The accumulation appeared similar for all the tissues studied except skin and hair, stomach and possibly the intestine, in which a relatively greater accumulation of ^{35}S was demonstrated when sulphite was fed. When [^{35}S]sulphited rat-serum protein (134–169 mg) was fed to rats by stomach tube, absorption and utilization of the protein was indicated by excretion of 40–55% of the label in the urine within 24 hr, 90% of this excreted radioactivity being in the form of sulphate. Radioactivity from this same sulphited protein (56 mg) injected into the jugular vein was retained within the body to a much greater extent (48%) than ingested protein label (7–17%) at 24 hr. The marked difference in the distribution of ^{35}S from sulphited rat-serum protein injected into the blood stream from that found with ingested free [^{35}S]sulphite suggests that sulphited plasma proteins may not be intermediates in the metabolism of ingested sulphite. Rats fed diets containing autoclaved, metabisulphite-treated casein for 7 wk grew as well as control animals fed diets containing autoclaved unsulphited casein.

INTRODUCTION

A recent paper from our laboratory was concerned with the fate of acute doses of sulphite in mice, rats and monkeys (Gibson & Strong, 1973). This work showed that most of the ingested sulphite could be readily metabolized to sulphate and eliminated in the urine within 24 hr in all three species. Attempts to saturate the sulphite-metabolizing pathways in the rat and thus produce free sulphite in the urine were unsuccessful, even with single oral doses as high as 400 mg SO_2/kg. No induction of sulphite oxidase could be detected after a single oral dose of 200 mg SO_2/kg nor after 50 or 200 mg SO_2/kg/day given for 30 days. While these studies showed that animals have a large capacity to metabolize ingested sulphite with the enzymes already present in their tissues, they provided little information regarding the effect of long-term, low-dose feeding, comparable to human experience.

Our first concern in the work reported in the present paper was whether ingested sulphite-sulphur accumulated in any particular tissue to a greater extent than sulphate-sulphur, as such a finding would indicate sulphite-specific sites of interaction. Secondly, questions are often raised concerning 'bound forms' of dietary sulphite. Information about the absorption and metabolism by rats of sulphited plasma protein, comparable to protein-bound sulphite in foods, has also been sought in the present work. Finally, the effect of autoclaved sulphited casein in rat diets was studied because of reported growth inhibition by this material (Bhagat & Lockett, 1964).

EXPERIMENTAL

Accumulation experiments

Groups of six male and six female albino rats of approximately 100 g body weight, obtained from the Holtzman Co., Madison, Wisc., were dosed by stomach tube daily for 14 days with either $Na_2{}^{35}SO_3$ or $Na_2{}^{35}SO_4$ purchased from New England Nuclear, Chicago, Ill. The doses consisted of 2·0 ml portions of 0·5% aqueous glucose solution containing either $Na_2{}^{35}SO_3$ plus cold $NaHSO_3$ to provide a dose of 50 mg SO_2/kg body weight, or $Na_2{}^{35}SO_4$ plus cold Na_2SO_4 to provide a dose of the same sulphur content. The rats were weighed every other day, the weights of the males and females were averaged separately and the doses were adjusted with cold Na_2SO_3 or Na_2SO_4, respectively, so as to maintain the same doses per kg body weight as nearly as possible throughout the experiment. The average doses of radioactivity each rat received are given in Tables 1 and 2. The actual amount administered to each animal was determined each day by analysing an aliquot of the isotopic solution used. Since $Na_2{}^{35}SO_4$ was purchased as a solution, the radioactive doses of sulphate were quite uniform from day to day. The $Na_2{}^{35}SO_3$, however, being stable only in the solid state, was more difficult to prepare in a consistent manner and the doses of radioactivity varied considerably (0·5–2·4 times the average).

The rats were fed *ad lib.* with Wayne Lab-Blox, purchased from Allied Mills, Inc., Chicago, Ill. At each of three intervals (2, 7 and 14 days) after the start of the experiment, two male and two female rats were killed by a blow on the head 24 hr after receiving their last dose. A 1 ml blood sample was taken for radioactivity analysis, the remaining blood being analysed as part of the 'carcass'. The tissues were removed, rinsed in 0·9% saline, blotted dry and weighed, with the exception of the skin and 'carcass', which were only weighed. The dissection of the brain included tissue from the olfactory bulb at the anterior end to just posterior to the cerebellum. The stomach and intestines were washed several times with 0·9% saline and the washings were discarded. The intestines were generally cut into smaller sections to facilitate washing, which was done with the aid of a large syringe while the tissue was expressed with the fingertips. The bladder was expressed to force urine out on to absorbent paper; this facilitated removal of the reproductive organs and decreased potential contamination from urine. The bladder was eventually excised, opened and washed prior to digestion with the 'carcass'. The male reproductive organs included the testes, epididymus and attached fat pad, the prostate, coagulating gland and seminal vesicles; the penis was not included. The uterus was excised just anterior to the bladder and removed as completely as possible. The 'carcass' contained all tissues not designated as being in other samples.

The various samples were dissolved separately in Pirie's reagent and prepared for isotopic analysis as previously described (Gibson & Strong, 1973). The radioactive counting was done on a Packard Model 3003 Tricarb Scintillation Spectrophotometer. The entire study was replicated once, so that two complete experiments were carried out with both sulphite and sulphate. The replicate experiments, designated A and B, were conducted at different times.

Preparation and feeding of [^{35}S]*sulphited rat-serum protein*

Blood for preparation of rat-serum protein was obtained from male albino rats by cardiac puncture. The blood was permitted to clot at room temperature for about 1 hr before being centrifuged for 10 min at full speed in an International Clinical Centrifuge. The

Table 1. *Tissue radioactivity accumulated by rats dosed daily with sodium [^{35}S]sulphate* by gastric intubation*

Tissue	Values for individual rats in experiment							
	A				B			
	Males		Females		Males		Females	
	Weight (g)	cpm . 10^{-3}	Weight (g)	cpm . 10^{-3}	Weight (g)	cpm . 10^{-3}	Weight (g)	cpm . 10^{-3}
Day 2 results								
Carcass	61·4	631	63·2	544	60·2	427	63·6	374
	62·9	630	65·6	590	64·4	398	63·0	441
Skin	15·9	144	20·6	165	15·8	93·2	17·8	87·7
	18·3	174	17·9	161	18·4	91·5	18·1	110
Intestine	7·17	72·9	6·28	59·6	7·07	43·0	6·95	34·9
	6·81	78·3	6·47	74·9	7·37	42·4	7·10	51·1
Liver	5·73	23·1	4·87	12·5	5·92	9·42	5·19	8·51
	5·23	22·6	5·07	22·3	6·24	10·3	5·27	11·7
Reproductive organs	1·81	9·66	0·695	2·53	1·77	5·48	0·685	1·41
	1·97	15·3	0·530	2·30	1·93	5·60	0·585	1·40
Brain	1·60	5·88	1·56	5·29	1·64	3·58	1·61	3·10
	1·53	5·95	1·65	5·55	1·60	3·26	1·53	2·36
Lungs	0·970	6·93	1·11	6·67	0·855	2·94	0·920	3·50
	1·04	7·17	0·920	6·28	1·90	4·92	0·910	4·11
Kidneys	1·18	12·0	1·02	10·3	1·21	7·54	1·10	6·05
	1·11	13·2	1·17	13·1	1·25	7·81	1·08	7·72
Stomach	0·695	8·53	0·810	7·92	0·795	5·03	0·890	4·62
	0·890	10·8	0·855	8·63	0·875	5·43	0·830	5·80
Heart	0·475	2·41	0·500	2·16	0·425	0·989	0·530	1·20
	0·520	2·75	0·555	2·62	0·430	1·16	0·460	1·30
Spleen	0·460	2·97	0·530	2·98	0·410	1·25	0·510	1·45
	0·475	3·78	0·575	4·88	0·455	1·60	0·495	2·11
Day 7 results								
Carcass	81·8	1045	63·1	1236	80·5	954	67·3	721
	83·9	1311	63·0	1110	82·7	897	67·0	814
Skin	26·7	326	19·6	330	26·8	278	20·8	204
	25·1	354	20·1	349	30·0	284	21·9	221
Intestine	7·94	106	6·76	107	8·51	68·4	7·05	58·6
	8·78	108	6·79	90·4	10·7	85·7	7·31	70·8
Liver	5·85	22·7	4·64	28·3	6·77	23·4	4·83	13·8
	5·58	32·1	4·44	20·6	7·05	23·8	4·88	17·6
Reproductive organs	2·56	19·0	0·860	5·23	2·61	14·8	0·675	3·53
	2·88	22·0	0·620	5·60	2·84	14·7	0·860	3·81
Brain	1·57	10·7	1·35	12·1	1·51	7·65	1·51	6·76
	1·53	10·5	1·36	12·3	1·47	7·72	1·53	5·92
Lungs	1·41	12·6	0·880	10·2	1·40	7·25	1·72	7·07
	1·44	14·6	0·880	9·42	1·12	7·14	1·11	6·80
Kidneys	1·32	16·4	1·10	20·6	1·52	15·0	1·19	10·6
	1·36	18·7	1·10	16·1	1·46	13·8	1·12	11·9
Stomach	1·01	14·6	0·760	12·1	1·03	10·3	0·970	6·97
	1·24	16·4	0·760	11·6	1·07	10·7	0·855	7·69
Heart	0·605	3·40	0·530	4·63	0·605	2·74	0·495	1·96
	0·715	4·71	0·490	3·39	0·625	2·74	0·590	2·82
Spleen	0·755	5·70	0·480	5·32	0·560	2·88	0·520	2·29
	0·645	5·84	0·510	4·02	0·740	3·22	0·640	5·70
Day 14 results								
Carcass	113	1827	104	1650	95·8	1124	94·7	1172
	108	1932	98·0	1633	104	1365	91·1	1223
Skin	35·7	561	30·9	603	30·5	360	27·8	422
	33·8	715	30·5	630	30·7	442	28·2	401
Intestine	8·68	129	8·74	118	10·1	93·5	8·79	88·4
	10·4	118	8·82	106	11·5	97·1	8·57	98·0
Liver	7·30	37·3	7·80	41·1	7·64	16·1	8·14	28·9
	9·78	45·9	7·72	39·0	9·62	29·6	6·92	29·8
Reproductive organs	4·60	35·8	2·09	11·7	3·40	22·2	1·20	5·13
	3·60	30·2	1·86	9·36	3·61	24·1	1·22	3·42
Brain	1·85	15·9	1·71	15·5	1·46	8·61	1·74	12·0
	1·51	15·6	1·62	14·7	1·43	10·5	1·53	11·7
Lungs	1·34	14·3	1·65	14·5	2·81	11·6	1·16	9·41
	1·71	15·6	1·18	11·6	2·04	10·8	1·42	9·86
Kidneys	1·67	20·1	1·61	20·1	1·54	13·4	1·47	14·9
	1·81	19·7	1·87	22·8	1·74	15·1	1·29	15·0
Stomach	1·33	16·5	1·29	15·8	1·07	10·7	1·13	10·7
	1·55	16·3	1·50	15·8	1·28	12·3	1·06	11·3

Table 1 (*cont.*)

Tissue	A Males Weight (g)	A Males cpm. 10^{-3}	A Females Weight (g)	A Females cpm. 10^{-3}	B Males Weight (g)	B Males cpm. 10^{-3}	B Females Weight (g)	B Females cpm. 10^{-3}
Heart	0·765	4·90	0·805	5·57	0·750	3·17	0·745	4·15
	0·815	6·07	0·795	5·61	0·795	4·11	0·630	3·43
Spleen	0·760	5·87	0·760	5·26	0·705	3·93	0·575	3·15
	0·815	6·82	0·720	5·03	0·830	5·63	0·580	3·82

*Total radioactivity administered to each rat by days 2, 7 and 14, respectively, was as follows: experiment A, males and females, 19·6, 65·6 and 130 × 10^6 cpm; experiment B, males, 14·7, 53·1 and 106 × 10^6 cpm, and females, 14·7, 53·2 and 103 × 10^6 cpm.

Table 2. *Tissue radioactivity accumulated by rats administered sodium [^{35}S]sulphite* by gastric intubation*

Tissue	A Males Weight (g)	A Males cpm. 10^{-3}	A Females Weight (g)	A Females cpm. 10^{-3}	B Males Weight (g)	B Males cpm. 10^{-3}	B Females Weight (g)	B Females cpm. 10^{-3}
Day 2 results								
Carcass	68·9	191	63·3	215	57·2	1069	53·5	1452
	67·7	147	60·0	186	61·8	1019	52·2	1266
Skin	17·9	80·6	18·5	77·9	15·5	523	15·8	491
	16·9	33·9	17·9	57·4	14·9	230	15·7	454
Intestine	7·67	20·7	7·05	26·5	7·88	203	5·50	299
	7·30	16·3	6·83	30·6	7·39	206	5·85	232
Liver	7·50	6·21	5·25	8·13	3·94	37·6	3·25	55·7
	6·87	4·00	4·26	9·02	3·97	37·6	3·38	56·4
Reproductive organs	2·22	2·94	0·680	0·980	1·13	12·6	0·380	4·89
	2·11	2·77	0·535	1·08	1·84	16·8	0·270	4·00
Brain	1·58	1·61	1·54	1·89	1·46	7·94	1·53	9·16
	1·41	1·67	1·59	2·21	1·57	8·28	1·47	11·2
Lungs	1·08	1·98	1·34	2·96	1·22	15·3	0·865	21·0
	1·34	1·94	0·960	2·73	1·27	16·6	0·775	15·6
Kidneys	1·40	3·73	1·15	4·39	1·08	23·7	0·940	31·8
	1·26	3·02	1·11	4·82	1·09	21·4	0·840	26·8
Stomach	0·890	3·57	0·910	5·76	0·840	33·2	0·880	65·8
	0·945	2·52	0·765	4·71	0·785	34·5	0·655	44·7
Heart	0·520	0·675	0·470	1·01	0·460	4·37	0·485	6·67
	0·470	0·690	0·490	0·990	0·515	3·73	0·520	7·06
Spleen	0·565	1·29	0·530	1·27	0·455	5·59	0·435	7·77
	0·415	0·860	0·560	1·53	0·510	5·73	0·475	7·71
Day 7 results								
Carcass	76·0	307	77·6	337	77·8	1983	78·0	1739
	79·6	368	81·6	351	85·2	1711	73·7	2144
Skin	24·6	215	24·4	107	23·4	612	26·5	769
	22·4	129	24·7	99·1	26·3	659	23·6	781
Intestine	8·39	28·7	8·34	30·7	8·44	164	6·05	152
	9·38	46·5	8·62	32·5	8·18	157	6·42	155
Liver	5·79	7·70	5·31	7·22	6·61	60·7	5·46	64·8
	6·22	12·3	5·88	6·05	5·37	51·7	5·02	51·9
Reproductive organs	2·93	6·45	1·43	2·74	2·43	32·7	1·74	22·6
	2·60	7·55	1·47	2·52	2·41	33·9	2·18	23·2
Brain	1·26	2·74	1·46	1·47	1·56	17·8	1·15	9·65
	1·44	3·41	1·55	2·77	1·56	16·1	1·57	16·2
Lungs	1·31	3·50	1·16	3·99	1·21	20·8	1·00	19·8
	1·06	4·55	1·00	2·95	1·21	19·5	0·935	17·4
Kidneys	1·32	5·83	1·04	4·98	1·34	26·7	1·29	32·1
	1·36	7·15	1·28	5·54	1·36	29·1	1·35	33·4
Stomach	1·24	5·95	1·02	5·71	0·980	28·1	1·00	31·6
	1·06	7·07	1·04	6·17	1·14	31·6	0·935	35·0
Heart	0·570	1·36	0·625	1·07	0·630	7·20	0·575	7·39
	0·665	1·59	0·580	1·03	0·630	7·18	0·630	7·04

Table 2 (*cont.*)

	Values for individual rats in experiment							
	A				B			
	Males		Females		Males		Females	
Tissue	Weight (g)	cpm . 10^{-3}	Weight (g)	cpm . 10^{-3}	Weight (g)	cpm . 10^{-3}	Weight (g)	cpm . 10^{-3}
Spleen	0·740	0·996	0·720	1·20	0·705	7·85	0·490	7·29
	0·730	2·01	0·600	1·19	0·625	8·30	0·620	7·78
			Day 14 results					
Carcass	112	447	100	579	110	2463	80·6	1732
	118	629	88·9	581	116	2394		
Skin	40·0	221	27·3	225	33·9	876	22·7	851
	38·6	281	25·8	159	37·6	1126		
Intestine	11·8	50·1	9·90	55·6	11·0	191	7·18	200
	11·7	78·0	8·84	40·3	11·3	222		
Liver	11·0	15·7	8·70	14·3	8·51	52·5	5·76	69·9
	12·3	18·6	7·65	9·85	10·5	89·1		
Reproductive organs	4·56	12·5	1·70	4·39	4·11	46·2	1·28	14·2
	4·68	14·7	2·00	2·84	3·87	41·7		
Brain	1·08	6·19	1·61	5·68	1·67	23·1	1·64	24·8
	1·77	6·23	1·57	5·22	1·89	29·3		
Lungs	1·15	4·39	1·76	6·00	1·55	25·7	2·91	46·4
	1·06	7·66	1·06	4·59	1·57	25·1		
Kidneys	1·94	9·33	1·68	9·32	1·77	30·6	1·24	30·9
	2·25	12·3	1·45	7·16	2·08	36·7		
Stomach	1·53	8·06	1·37	10·8	1·31	27·2	1·03	52·2
	1·47	14·3	1·28	6·42	1·30	29·5		
Heart	0·845	1·90	0·670	2·06	0·865	7·50	0·700	9·02
	0·950	2·51	0·635	1·42	1·00	12·0		
Spleen	0·760	1·29	0·575	2·05	0·760	7·54	0·675	13·7
	1·06	3·40	0·575	1·96	0·775	8·51		

*Total radioactivity administered to each rat by days 2, 7 and 14, respectively. was as follows: experiment A, males. 5·12, 22·1 and 53·8 × 10^6 cpm and females, 5·12, 22·6 and 56·2 × 10^6 cpm; experiment B. males, 31·8, 92·1 and 192 × 10^6 cpm and females, 31·8, 102 and 203 × 10^6 cpm.

supernatant fluid was dialysed against distilled water for 24 hr and again centrifuged to remove any precipitate formed during dialysis. The protein content of the supernatant was determined by the method of Lowry, Rosebrough, Farr & Randall (1951) using crystalline bovine serum albumin from Sigma Chemical Co. (St. Louis, Mo.) as a standard.

The protein was sulphited according to Procedure I, described by Cole (1967), except that radioactive $Na_2{}^{35}SO_3$ (New England Nuclear, Boston, Mass.) was added to the protein–copper solution prior to the addition of 0·7 M-Na_2SO_3. In the first of two preparations, 1 g protein was treated with 0·5 mCi $Na_2{}^{35}SO_3$ which had been dissolved in 2·0 ml of 0·5% glucose. This preparation yielded a protein with a specific activity of 7·5 × 10^3 cpm/mg. In the second preparation, 4 g protein was treated with 1·0 mCi $Na_2{}^{35}SO_3$ dissolved in 5·0 ml water and yielded a protein containing 4·1 × 10^3 cpm/mg. The labelled dialysed proteins were concentrated by freeze-drying followed by dissolution in a minimal amount of distilled water.

Chromatography of 0·3 ml of one of the above solutions containing 12·7 mg ^{35}S-labelled protein on a 1 × 21·5 cm column of Bio Gel P-100 equilibrated with 0·1 M-phosphate, pH 7·0, showed only one peak of absorption at 280 nm. This peak eluted between ml 4 and 8 and contained all the radioactivity added to the column. Chromatography on this same column of 0·3 ml 5 M-β-mercaptoethylamine produced a 280 nm absorption peak between ml 12 and 21; this absorption was apparently due to the end absorption of this compound. When the above amounts of β-mercaptoethylamine and protein were mixed,

incubated at room temperature for 30 min and subsequently chromatographed as indicated above, two 280 nm absorbing peaks appeared, one between ml 3 and 9 due to protein and one between ml 12 and 21, due to β-mercaptoethylamine, but analysis of the radioactivity indicated that 91·1% of the ^{35}S was now associated with the β-mercaptoethylamine peak. This is supporting evidence that the linkage between the [^{35}S]sulphite and the protein was of the desired *S*-sulpho type since this linkage is readily broken by an excess of small molecular weight sulphydryl compounds.

In each of two experiments, 12 male albino rats (Holtzman), weighing 100–110 g, were fed the above ^{35}S-labelled rat-serum protein by stomach tube. In the first experiment, each animal received 3·0 ml of solution containing 134 mg protein with a radioactivity content of $9{\cdot}3 \times 10^5$ cpm. In the second experiment, each animal received 3·0 ml of a solution containing 169 mg protein with a radioactivity content of $6{\cdot}7 \times 10^5$ cpm. Food and water were provided *ad lib.* over the experimental period. Groups of three rats were killed 24 and 48 hr after the administration, during which periods urine and faeces were collected. The remaining two groups of three rats were killed 1 and 2 wk after dosing. Analysis of the carcass, urine and faeces was carried out as previously described (Gibson & Strong, 1973). Urine from the animals killed after 1 and 2 wk was collected only over the first 24 hr after administration.

Urine was collected at room temperature in flasks containing either 8·0 ml 0·001 M-phosphate, pH 7·0, or 100 ml 0·02 M-*N*-ethyl maleimide (NEM) in ethanol, or in empty flasks maintained in solid CO_2. All samples were then stored at $-20°C$ until analysed by chromatography or treated with barium chloride.

The urine samples obtained in this way were examined by descending chromatography, carried out on Whatman No. 1 chromatography paper using isopropanol–water (7:3, v/v) as described by Nakamura & Sato (1962). Generally, 0·1 ml aliquots of the urine collected over solid CO_2 or in phosphate buffer were chromatographed. Urine collected in ethanolic NEM produced a fine white precipitate. A representative 1·5 ml aliquot of the suspension was allowed to settle, and the liquid portion was applied in small portions to a narrow band at the origin of a 4 cm strip of chromatography paper. The solid material was then dissolved in a few drops of water and also applied to the same area of the paper. The developed chromatograms were generally scanned for radioactivity on a Vanguard Model 880 Low-Background Autoscanner. Quantitation was achieved by cutting the chromatogram into 1 cm strips, which were each placed in a vial containing 5 ml toluene scintillation fluid. Radioactivity was then determined in a Packard Tri-Carb liquid scintillation spectrometer. Chromatographic standards included [^{35}S]sulphate and ^{35}S-labelled and unlabelled *S*-sulphocysteine prepared according to the method of Segel & Johnson (1963). Visualization of *S*-sulphocysteine was achieved by using a ninhydrin spray (62 mg ninhydrin and 0·25 ml collidine in 25 ml acetone).

The authenticity of the [^{35}S]sulphocysteine standard was determined by descending paper chromatography before and after performic acid oxidation. To do this a 7·5 μl aliquot of a saturated aqueous solution of [^{35}S]sulphocysteine was added to a mixture consisting of 40 μl 88% formic acid and 100 μl 30% hydrogen peroxide. After 30 min incubation at room temperature, the entire reaction mixture was chromatographed. Analysis of the chromatograms indicated that before oxidation the only spot found was radioactive and reacted positively with ninhydrin. After oxidation, the position of the radioactivity on the chromatogram was separated from the ninhydrin-positive area and had the same R_F as sulphate; the ninhydrin-positive spot chromatographed with the same R_F as cysteic

acid. No other products were observed. Sulphate and cysteic acid are the two products expected from the oxidation of *S*-sulphocysteine.

Aliquots of urine (2·0 ml) to be treated with barium chloride were acidified to pH 1 with 1 N-HCl in a graduated centrifuge tube. To the acidified urine was added 4 μmols of unlabelled Na_2SO_4 and enough water to bring the volume to 5·0 ml. A 0·5 ml aliquot was taken for determination of isotope content and the remainder was heated to boiling on a boiling water-bath. To this mixture was added, dropwise with stirring, 2·0 ml of barium chloride solution containing 80 μmols barium. The mixture was stirred for 10 min in the boiling water-bath and permitted to cool overnight. In the morning, the mixture was centrifuged for 10 min at full speed in an International Clinical Centrifuge and the supernatant was sampled for ^{35}S content.

Intravenous injection of [^{35}S]sulphited rat-serum protein

Twelve male albino rats of approximately 100 g body weight were anaesthetized with ether. A small incision was made just above the left clavicle of each and the skin was parted to expose the left external jugular vein. An injection of 1·0 ml [^{35}S]sulphited rat-serum protein prepared as described above, pH 6·0—6·5, was made directly into the jugular vein over a period of 2–10 min. This injection contained 56·3 mg protein and a radioactivity level of $1{\cdot}29 \times 10^5$ cpm. After injection, the wound was closed with nickel-silver clips. Food and water were provided *ad lib.* throughout the experimental period. Groups of three rats were killed 24 and 48 hr after the injection, during which periods urine and faeces were collected, and the remaining two groups of three rats were killed 1 and 2 wk after injection. Separate analyses for the ^{35}S content of the liver, spleen, blood (a 1·0 ml aliquot) and the remainder of the carcass, as well as the urine and faeces, were carried out.

Sulphite in solid diets

Powdered desiccated whole liver, dried brewers yeast and salt mixture no. 2 (USP XII) were purchased from General Biochemicals Corp. (Chagrin Falls, Ohio). Casein and anhydrous sodium metabisulphite ($Na_2S_2O_5$) were obtained from the Sigma Chemical Co. (St. Louis, Mo.). Corn starch was purchased from Best Foods (Englewood Cliffs, N.J.) and corn oil was obtained from the Beaver Dam Wholesale Co. (Beaver Dam, Wisc.). Female albino rats (50–60 g) were obtained from Sprague-Dawley (Madison, Wisc.).

The day before the diets were prepared, a portion of casein was autoclaved at 18 psi for 4 hr, and another portion, containg 0·6% (w/w) admixed solid sodium metabisulphite, was similarly treated. After cooling, the solid blocks of material were broken and powdered separately in a ball mill. These portions were the only casein used in the diets. Next day, four diets were prepared, with the following percentage compositions: Diet A—corn starch 61·2, casein 18·4, corn oil 6·1, powdered desiccated whole liver 5·1, brewers yeast 5·1, salt mixture no. 2 (USP XII) 4·1%; diet B—the same as diet A, but with metabisulphite-treated casein replacing untreated casein; diet C and D—as diets A and B, respectively, except that the casein amount was doubled by addition of untreated casein, while the amounts of the other components remained constant. Thus, the casein level in diets C and D was 30·6% of the total diet, 50% of this level being metabisulphite-treated in the case of diet D. The diets were sealed in polyethylene bags and stored in the dark at room temperature. Sufficient diet was prepared at one time to last the whole experiment. The experiment was initiated on the day the diets were prepared.

Rats (24), housed in individual cages, were separated into four groups of six animals. Each group was fed one of the diets for a period of 47 days (experiment A). Water and diet were supplied *ad lib.* and the animals were weighed weekly. Each rat received 0·5 ml cod-liver oil once weekly by stomach tube. The entire experiment was repeated once, the replicate experiment (B) being conducted at a different time and continuing for 49 rather than 47 days.

RESULTS AND DISCUSSION

The complete data obtained in the sulphate and sulphite accumulation experiments are presented in Tables 1 and 2, respectively. The total radioactivity administered and the weight and radioactivity of individual tissues from each animal killed at the end of the designated experimental periods are included. The possibility existed that the observed tissue radioactivity might have been materially affected by isotopically labelled blood contained in the tissues, and to estimate the magnitude of this factor, 1 ml of blood from each test animal was analysed separately. Assuming that a 100 g rat has 5 ml of blood, it was calculated that the 4 ml remaining in the carcass could have contributed only 0·076–0·26% of one daily dose to the total body radioactivity. For a 200 g rat, the corresponding range was 0·17–0·59%. In only two instances did the values fall outside these ranges, namely for the day 2 females in the sulphite experiment B, the calculated values for these rats being 0·37 and 0·48%, respectively.

Figures 1a and b show the rate of whole-body accumulation of ^{35}S from sulphate and sulphite, expressed either as a percentage of one daily dose averaged to the time the animals were killed (Fig. 1a) or as a percentage of the total cumulative dose of ^{35}S to the same time (Fig. 1b). Except for the day 14 sulphite data, which is for seven, each point represents the mean for eight animals, four male and four female, with the standard error of the mean indicated.

The average rate of sulphite accumulation for days 1–2 was 6·05% of the daily dose/day, while for days 3–7 it was 1·40% and for days 8–14, 0·70%. It appears that some reaction or process is being saturated during the initial dosing period, and thereafter, a second process becomes rate-limiting. This contrasts with the accumulation of sulphate, which for days 1–2 was 4·32% of the daily dose/day, for days 3–7 was 1·73%, and for days 8–14 was 1·23%. The day 2 data for the two cases are statistically different by Student's t test ($P < 0{\cdot}001$).

Figure 2 shows the relative distribution of radioactivity among the tissues of these same animals calculated as a percentage of total body radioactivity in a given tissue divided by the percentage of total body weight contributed by that tissue. This value for the whole animal, therefore, is 1·00, corresponding to the average rate of accumulation for the entire body. Values greater than 1·00 indicate a faster than average accumulation and *vice versa*. Each bar represents the average of the data for eight rats as presented in Tables 1 and 2.

The more rapid initial uptake of sulphite involves the stomach and intestines, probably because these tissues are the first site of contact with sulphite. The rate of uptake of sulphite is not only greater in these two tissues in the early part of the experiment, but also remains high in the stomach throughout the experimental period (Fig. 2). This may be attributable in part to the initial high concentration in the stomach, which is reflected in the sulphate data as well. However, although the uptake of sulphate into the stomach is high at first, it approaches the body average by day 7, whereas at this time, sulphite is

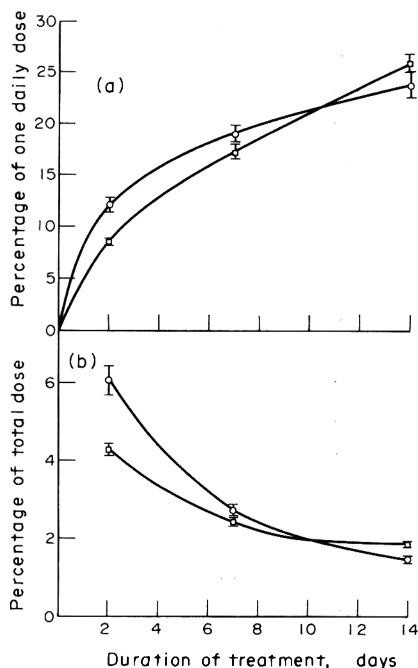

Fig. 1. Whole-body retention of ^{35}S by rats fed ^{35}S-labelled sodium sulphite (○) or sodium sulphate (□) at levels equivalent to 50 mg SO_2/kg body weight/day for 2, 7 or 14 days. Each point represents the mean value for eight animals, four males and four females, except for the sulphite data for day 14 which represents only seven rats. The standard error of the mean is indicated in each case, and the ^{35}S accumulation is expressed (a) as a percentage of one daily dose of ^{35}S averaged to the time the rats were killed and (b) as a percentage of the total cumulative dose of ^{35}S to the same time.

still accumulating in the stomach at a faster than average rate. Evidently, some factor other than concentration is involved in this organ.

The skin and hair are also involved in the initial reactions peculiar to sulphite, a finding which may be a consequence of the high cystine content of hair. Using germ-free rats, Huovinen & Gustafsson (1967) showed that sulphate and sulphite cannot be utilized for the formation of cysteine except by prior metabolism by the micro-organisms in the gastrointestinal tract. Also it seems likely that the lower oxidation level of sulphite-sulphur may facilitate its incorporation into cystine.

Accumulation of ^{35}S from sulphite in the stomach, intestines and skin and hair was more variable among individual animals than that from sulphate, probably because of the food content of the digestive tract at the time of dosing, an uncontrolled variable. The higher reactivity of sulphite could well lead to greater reaction with food constituents, and thus alter its reaction with stomach or intestinal tissues. Alternatively, utilization or reaction might be dependent upon concentration and/or pH, both of which could vary according to the stomach contents at the time of dosing.

Sulphite and sulphate are similarly distributed throughout the body with the exception of the specific tissues mentioned above. This can most easily be explained by oxidation of the sulphite to sulphate either in the intestinal tract or immediately after absorption. If sulphite were absorbed intact, the circulation would carry it to the liver, where sulphite oxidase is abundant as shown by MacLeod, Farkas, Fridovich & Handler (1961). Our own

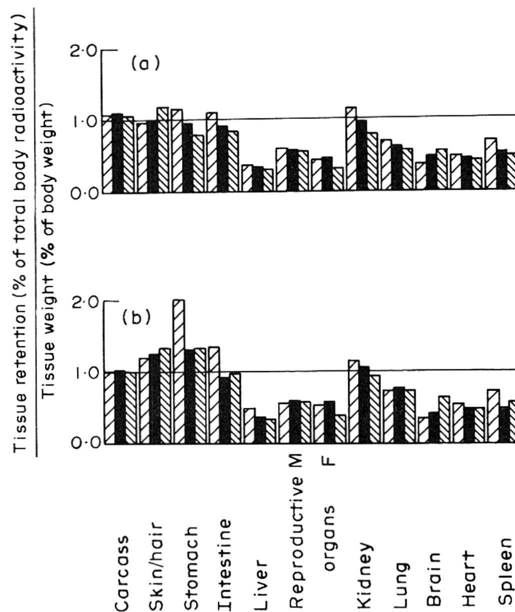

Fig. 2. Relative accumulation of ^{35}S in various tissues of rats fed sodium [^{35}S]sulphate (a) or sodium [^{35}S]sulphite (b) at levels equivalent to 50 mg SO_2/kg body weight/day for 2 (▨), 7 (■) or 14 (▩) days and then killed.

previous work (Gibson & Strong, 1973) showed that the rat could effectively oxidize doses of sulphite eight times higher than those used in the present experiments, so a similar distribution for both sulphate and sulhpite was not unexpected.

Since sulphites react readily with the disulphide linkages in proteins to produce *S*-sulphocysteine residues, it is probable that such modified proteins are present in sulphited foods. The metabolic fate of such bound forms of sulphite was investigated in the present study by both oral and iv administration to rats of [^{35}S]sulphited rat-serum protein preparations. The results of two feeding experiments are shown in Table 3, which gives the percentage of the administered ^{35}S subsequently found in the urine, faeces and carcass of the test animals. That the sulphited protein was absorbed and metabolized to a considerable extent is indicated by the appearance in the urine of 42–62% of the administered radioactivity after 24–48 hr. The rather large difference in the extent of utilization in the two experiments was apparently not due to experimental error since the overall isotope recovery was satisfactory (Table 3). The variation may have been related to a seasonal difference in the animals, as the replicate experiments were carried out in February and June, or to differences in the composition of the two protein preparations fed.

Nevertheless, in both cases, the actual amount of protein absorbed from the intestinal tract was only a part of the dose administered. A male laboratory rat of 110 g body weight requires 3·0 g protein/day for normal growth, 1·8 g of which is digestible (Committee on Nutrition, 1962). The amount of sulphited protein used in these experiments (134 and 169 mg/rat) was only a small fraction of this amount and yet only 45–70% of the ^{35}S that it contained was found in the carcass and urine. Apparently, there is some difficulty in the utilization of this protein, or it may be incorporated into micro-organisms which remain in the faeces.

Table 3. *Disposition of ^{35}S given to rats in a single oral dose of [^{35}S]sulphited protein*

Group no.	Time after dosing (days)	Experiment	Total radioactivity (% of administered dose) in: Urine	Faeces	Carcass	Recovery (%)
1	1	A	55·5 (52–58)	27·9 (26–31)	13·3 (11–17)	96·8 (94–100)
		B	43·3 (42–44)	45·1 (41–49)	10·7 (7–16)	99·1 (98–101)
2	2	A	62·4 (58–66)	28·6 (25–32)	5·0 (4·6–5·2)	96·1 (95–98)
		B	42·9 (42–43)	50·6 (50–52)	2·6 (2·4–2·8)	96·2 (95–97)
3	7	A	—	—	2·4 (1·7–3·3)	—
		B	—	—	1·4 (1·3–1·5)	—
4	14	A	—	—	1·6 (1·0–2·6)	—
		B	—	—	0·88 (0·7–1·1)	—

Values are given as means (with ranges in parentheses) for groups of three rats (body weight 100–110 g). Doses given to rats were: experiment A, 134 mg sulphited protein containing $9·3 \times 10^5$ cpm; experiment B, 169 mg sulphited protein containing $6·7 \times 10^5$ cpm.

The form in which the ^{35}S existed in the urine of these test animals was investigated in several ways, as summarized in Table 4. Where no treatment is indicated, samples, collected as described, were treated directly with barium chloride. Urine samples subjected to paper chromatography prior to precipitation are indicated. The chromatographic strips were scanned to locate the radioactive peak which was then eluted from the paper with water and precipitated with barium. Only one major radioactive excretion product was observed irrespective of the method of collection. This product co-chromatographed with inorganic [^{35}S]sulphate. There was no discernible accumulation of radioactivity at the R_F of *S*-sulphocysteine on these chromatograms. The sample chromatographed on Bio Gel P-100 was eluted from the column under the same conditions used for chromatography of [^{35}S]sulphited protein, and the fractions containing radioactivity were pooled and concentrated prior to barium chloride precipitation. All samples, except those chromatographed on paper, showed a small (9–12%) fraction of radioactivity not precipitable by barium. However, the major portion of radioactivity (88–91%) was shown by its precipitation to be inorganic sulphate. Subjecting urine to paper chromatography separated other

Table 4. *Barium precipitation of urine from rats given a single oral dose of [^{35}]sulphited protein*

Collection method	Treatment before precipitation	Total radioactivity in treated urine (cpm)	Urinary radioactivity not precipitated by barium: cpm	% of total
Phosphate	None	13310	1199	9
	Bio Gel P-100	9830	949	10
	Paper chromatography	7760	171	2
Solid CO_2	None	7120	826	12
	Paper chromatography	8330	42	1
Ethanolic-NEM	None	4260	381	10

Urine samples were collected from rats for 24 hr after dosing either at room temperature in flasks containing 8·0 ml 0·001 M-phosphate (pH 7·0) or 100 ml 0·02 M-*N*-ethyl maleimide (NEM) in ethanol, or in empty flasks maintained in solid CO_2. Aliquots of 2·0 ml were treated with barium chloride (see Experimental). After precipitation and centrifugation, the radioactivity content of the supernatant was determined.

minor radioactive components from the sulphate and resulted in complete (98–99%) precipitation of radioactivity from the eluted sulphate peak.

These experiments demonstrate that the major radioactive excretion product produced by rats fed [^{35}S]sulphited protein is inorganic sulphate. This finding implies that the *S*-sulphocysteine moiety and the protein fed can be degraded *in vivo* to yield not only inorganic sulphate, but presumably also cysteine, although the latter is not a proven product. The cleavage of the *S*-sulphocysteine moiety could take place in the acid environment of the stomach, in the micro-organisms of the intestinal tract, or within the tissues of the animal; this cannot be determined from the present data. It is apparent, however, that the sulphite produced from the cleavage of the *S*-sulphocysteine residues is oxidized prior to excretion, since otherwise the urine collected in ethanolic-NEM would have contained the sulphite-NEM reaction product, a sensitive test for sulphite (Gibson & Strong, 1973).

It was proposed by Gunnison & Benton (1971) that reaction of sulphite with constituents of blood plasma or serum might protect body tissues from detrimental effects that might otherwise be caused by high concentrations of free sulphite. Their data indicate that most of the reactivity of the plasma toward sulphite resides in the non-dialysable serum fraction, probably the proteins, and that the equilibrium for the reaction at physiological pH strongly favours sulphited protein over free sulphite. Therefore, most free sulphite entering the blood presumably reacts with serum proteins and circulates until eliminated through normal metabolic pathways.

The distribution of ^{35}S after injection of [^{35}S]sulphited protein directly into the blood stream of four groups of rats via the external jugular vein is shown in Table 5. At the end of 24–48 hr, approximately 50–66% of the administered ^{35}S was excreted, predominantly in the urine; after this time, the rate of excretion slowed considerably, so that even after 2 wk, 5–7% still remained in the body. The liver and spleen alone accounted for about 50% of the total radioactivity found in the entire body. The concentration of isotope by these two tissues is shown more clearly in Table 6, where the relative accumulation is seen to be 10–20 times as great as for the remainder of the body on a weight basis. The initial concentration persists throughout the duration of the experiment, although the liver appears to be able to eliminate the ^{35}S at a slightly faster rate than either the spleen or the remainder of the carcass (Table 6). Blood analyses showed a rapid removal of sulphited protein from the circulation, inasmuch as less than 0·2% of the dose was found in a 1·0 ml sample taken 24 hr after injection and less than 0·1% after 48 hr.

There is a marked difference in radioactivity distribution between rats dosed with [^{35}S]sulphited rat-serum protein injected directly into the circulation and those dosed orally with free [^{35}S]sulphite. When sulphited rat-serum protein was injected, the major accumulation appeared in the liver and spleen as described above, but this was not observed when free sulphite was fed orally. This difference may be explained in several ways. The sulphited protein prepared *in vitro* probably differs in the extent of sulphite addition when compared with that likely to be formed *in vivo*. In the *in vitro* preparation, cupric ions are added to the reaction mixture in order to oxidize free sulphydryl groups so that they may then react with sulphite (Cole, 1967). Under these circumstances and in the relatively high salt concentration present, it is possible partially or completely to unravel the tertiary structure of a protein. The extent of this unravelling would be dependent upon the explicit internal structure of the protein itself. Generally, however, most of the half-cystine residues present could be converted to the *S*-sulphonate form. By this mechanism, conformational changes may be produced in sulphited proteins prepared *in*

Table 5. *Fate of* [^{35}S]*sulphited protein after iv injection into rats*

Group no.	Time after dosing (days)	Total radioactivity (% of administered dose) in					Recovery (%)
		Liver	Spleen	Carcass	Urine	Faeces	
1	1	23·4 (22–25)	1·30 (1·2–1·4)	23·4 (22–25)	37·5 (33–45)	15·7 (11–22)	101·4 (96–104)
2	2	17·5 (17–19)	1·00 (0·7–1·4)	18·4 (16–23)	45·5 (39–50)	21·8 (19–26)	104·2 (103–105)
3	7	6·79 (6·5–7·1)	0·45 (0·41–0·53)	7·39 (7·1–7·6)	—	—	—
4	14	1·99 (1·7–2·2)	0·21 (0·20–0·23)	3·46 (2·9–4·4)	—	—	—

Values are expressed as means (with ranges in parentheses) for groups containing three animals. Each animal received 56·3 mg [^{35}S]sulphited protein containing $1{\cdot}29 \times 10^5$ cpm.

Table 6. *Relative accumulation of ^{35}S from sulphited protein after iv injection into rats*

Group no.*	Tissue	Mean weight† (g)	Mean weight (% of total)	Radioactivity (cpm as % of body total)	Relative accumulation‡
1	Carcass	108 (105–111)	93·9	48·6	0·52
	Liver	6·49 (6·0–6·8)	5·63	48·7	8·65
	Spleen	0·54 (0·50–0·57)	0·47	2·70	5·74
2	Carcass	118 (114–120)	94·6	49·6	0·52
	Liver	6·21 (5·7–6·8)	4·99	47·7	9·56
	Spleen	0·57 (0·51–0·66)	0·46	2·75	6·04
3	Carcass	146 (144–149)	94·1	50·5	0·54
	Liver	8·68 (7·2–9·7)	5·60	46·4	8·29
	Spleen	0·52 (0·48–0·56)	0·34	3·12	9·18
4	Carcass	189 (179–196)	94·8	60·7	0·64
	Liver	9·37 (8·9–9·9)	4·72	35·5	7·52
	Spleen	0·88 (0·67–1·11)	0·44	3·75	8·54

*Groups correspond to those given in Table 5.
†Ranges are given in parentheses.
‡Calculated from cpm expressed as % of body total/tissue weight expressed as % of total weight.

vitro, which would probably be irreversible when these same proteins are diluted with the constituents of whole blood *in vivo*. The nature of the sulphite-binding moiety of blood has not yet been clearly defined, nor has the possible extent of its sulphitolysis *in vivo* been investigated. It is therefore not possible to compare the effect of chemical and metabolic sulphitolysis of rat-serum proteins on their structural integrity at the present time. Nevertheless, if the sulphited proteins as prepared *in vitro* do differ significantly from proteins naturally suphited *in vivo*, they could appear as foreign proteins to the immune system and result in the observed accumulation in the liver and spleen.

If the quantity of sulphite fed in the accumulation experiments was too great for the liver to metabolize, any excess could have been absorbed by other tissues and preferential accumulation in the liver could have been obscured. The dose in the accumulation studies (50 mg SO_2/kg) was 17 times greater than in the injection experiments (3 mg SO_2/kg). However, the radioactivity present in the liver at 48 hr corresponded in the accumulation experiments (experiment A, 0·013 mg SO_2; experiment B, 0·015 mg SO_2), to only about 25% of that in the injection experiments (0·05 mg SO_2). Thus, in the accumulation experiments, the capacity of the animals' livers for metabolizing sulphite was not exceeded.

Our accumulation data comparing sulphate and sulphite indicate that the radioactivity found in the majority of tissues may simply be the result of sulphate metabolism, and one may question whether or not free sulphite ever gets into the blood at all from dietary sources, or at least past the liver. If sulphite were oxidized to sulphate before leaving the intestinal tract, it would preclude the possibility of sulphonate formation with blood proteins. On the other hand, if sulphite does enter the blood stream, protein sulphitolysis is probably not as extensive as that produced in our *in vitro* preparation, for if it were, one would observe significant accumulation in both the liver and spleen, which is not the case.

The growth of rats fed for a 7-wk period on diets in which the casein had been mixed with sodium metabisulphite and autoclaved (diets B and D) was as rapid as that of control animals fed autoclaved unsulphited casein (diets A and C) in the replicate experiments,

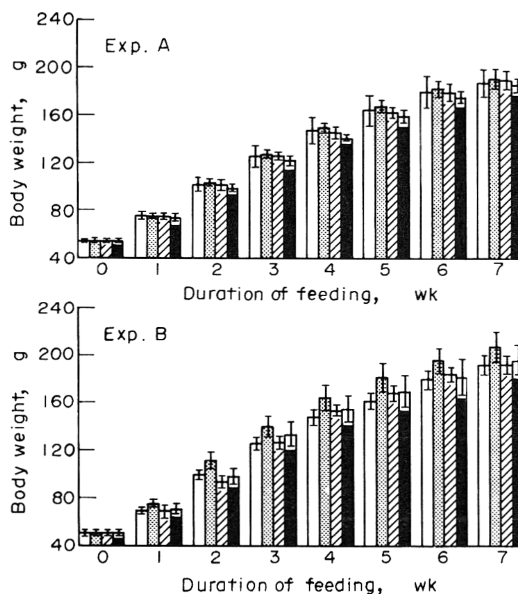

Fig. 3. Growth of rats fed in two experiments (A and B) on solid diets containing autoclaved casein or autoclaved metabisulphite-treated casein, as follows: Diet A (□), 18·4% casein; diet B (▨), 18·4% sulphited casein; diet C (▨), 30·6% casein; diet D (■), 15·3% casein plus 15·3% sulphited casein. Each bar represents the mean body weight for six female rats, except that for diet D in experiment B, which represents only five. The standard error of the mean is indicated for each weight measurement. Experiment A was terminated at 47 days instead of 49.

as shown in Fig. 3. No evidence was obtained that toxic substances were formed by autoclaving casein in the presence of 0·6% sodium metabisulphite. The majority of rats fed diet C or D in both experiments developed diarrhoea by the end of wk 1. After this time, the diarrhoea was less severe, but persisted for the remainder of the experimental period. Nevertheless, this had no significant effect upon the growth of the affected animals.

Our objective in carrying out these last experiments was to check the findings of Bhagat & Lockett (1964), who reported a 50% reduction in the growth of female rats fed sulphited autoclaved casein. It appeared that in their experiments the reported growth inhibition might have been due either to the development of toxicity or to the loss of some needed nutrient from the casein autoclaved with metabisulphite, and it was to check the second possibility that diets C and D were fed. However, although their experimental protocol was carefully followed, no growth-inhibiting effect at all was observed in the present work, and we have no explanation of the difference between our results and those of Bhagat & Lockett (1964).

REFERENCES

Bhagat, B. & Lockett, Mary F. (1964). The effect of sulphite in solid diets on the growth of rats. *Fd Cosmet. Toxicol.* **2**, 1.

Cole, R. D. (1967). Sulphitolysis. In *Methods in Enzymology*. Vol. XI. Edited by C. H. W. Hirs. p. 206. Academic Press, New York.

Committee on Nutrition (1962). Nutrient requirements of domestic animals. 10. Nutrient requirements of laboratory animals. National Academy of Sciences–National Research Council Publ. 990, Washington, D.C.

Gibson, W. B. & Strong, F. M. (1973). Metabolism and elimination of sulphite by rats, mice and monkeys. *Fd Cosmet. Toxicol.* **11**, 185.

Gunnison, A. F. & Benton, A. W. (1971). Sulfer dioxide: Sulfite. Interaction with mammalian serum and plasma. *Archs envir. Hlth* **22**, 381.
Huovinen, J. A. & Gustafsson, B. E. (1967). Inorganic sulphate, sulphite and sulphide as sulphur donors in the biosynthesis of sulphur amino acids in germ-free and conventional rats. *Biochim. biophys. Acta* **136**, 441.
Lowry, O. H., Rosebrough. Nira J., Farr, A. L. & Randall, Rose J. (1951). Protein measurement with the Folin phenol reagent. *J. biol. Chem.* **193**, 265.
MacLeod, R. M., Farkas, W., Fridovich. I. & Handler, P. (1961). Purification and properties of hepatic sulfite oxidase. *J. biol. Chem.* **236**, 1841.
Nakamura, T. & Sato, R. (1962). Accumulation of *S*-sulphocysteine by a mutant strain of *Aspergillus nidulans*. *Nature, Lond.* **193**, 481.
Segel, I. H. & Johnson, M. J. (1963). Synthesis and characterization of sodium cysteine-*S*-sulphate monohydrate. *Analyt. Biochem.* **5**, 330.

Fd Cosmet. Toxicol. Vol. 12 pp. 641-650. Pergamon Press 1974. Printed in Great Britain

SHORT-TERM TOXICITY OF QUILLAIA EXTRACT IN RATS

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Abstract—Quillaia extract was fed to groups of 15 male and 15 female rats for 13 wk at dietary concentrations of 0 (control), 0·6, 2·0 or 4·0%. There was a transitory reduction in the rate of body-weight gain, associated with a reduced intake of food and water, but by the end of the study the weights of the treated rats did not differ significantly from those of the controls. The feeding of quillaia saponin did not affect the results of haematological examinations (including erythrocyte fragility tests in hypotonic saline), serum and urine analyses, renal concentrating ability or the urinary cell excretion. The relative liver weight was reduced in males given 2·0 or 4·0% quillaia and the relative stomach weight was increased in both sexes at the same levels. No histopathological effects attributable to treatment were found. The no-untoward-effect level in this study was 0·6% of the diet, equivalent to an intake of approximately 400 mg/kg/day.

INTRODUCTION

Saponins are glycosides, usually nitrogen-free, consisting of a sapogenin and a sugar. The sapogenin may be either a steroid or triterpene. The commonest sugar moiety is glucose, although Coulson (1958) identified, in addition, arabinose, xylose, rhamnose and galactose when examining the saponins from lucerne, white clover and Quillaja. Jackson & Shaw (1959) identified a similar range of sugars in alfalfa saponins. The material chosen for the present studies (quillaia extract) was a triterpenoid saponin (Cheeke, 1971), in which two major sapogenins have been identified as well as one minor one and possibly a fourth, present only in trace amounts (Coulson, 1958).

This group of chemicals is found in over 400 species of plants including some, such as spinach, beetroot, sugar beet, asparagus, alfalfa, clover and soya, of importance as human or animal foods. They have been widely studied, particularly in relation to their growth-promoting effects and in relation to bloat production in ruminants. However, relatively little is known about the particular material used in the present work and it is questionable whether general conclusions can be drawn from studies with other saponins in view of their chemical diversity.

Saponins have amphipathic properties because of the water solubility of the carbohydrate moiety and lipid solubility of the sapogenin and are therefore used as emulsifiers and foaming agents, particularly in soft drinks, where the usage levels may be up to 300 ppm. They are also present in some flavours, such as sarsaparilla and liquorice (Merck Index, 1960), and are used in confectionery, toiletry preparations and pharmaceuticals.

As far as the use of quillaia extract in food in concerned, it is permitted at present in the UK under the Emulsifiers and Stabilisers in Food Regulations (Statutory Instrument 1962, no. 72) although specifically excluded from the definition of emulsifiers and stabilisers. However, a report of the Food Additives and Contaminants Committee (1970 & 1972) recommended its future inclusion in the permitted list, with the provisions that it

should be restricted to material of British Pharmacopoeia standard and used in soft drinks at not more than 200 ppm. It was classified as provisionally acceptable for use in food but a need for further information, including data from long-term studies and studies on possible intestinal irritation, was indicated.

Quillaia saponin has been shown to be degraded in the intestine. After oral doses in dogs two sapogenins are excreted in the faeces (Bäck, 1917), and Ewart (1931) has stated that the liberation of the sapogenins could be brought about by trypsin, diastase and pancreatic juice. However, Gestetner, Birk & Tencer (1968) were not able to confirm this in mice given soya saponin as they found no release of sapogenin in the small intestine. The probability is that micro-organisms in the gastro-intestinal tract are important in the degradation of saponins. This has been investigated in cattle in relation to bloat formation, and Guticrez & Davis (1962) were able to isolate saponin-digesting bacteria from the rumen of cattle fed on pastures carrying plants of high saponin content. Their work suggests that the bacteria utilize the carbohydrate moiety, leaving the insoluble sapogenin. In rats, mice and hens, Gestetner *et al.* (1968) found that soya-bean saponin was degraded only in the caecum and colon. They demonstrated that this required the presence of the micro-organisms and they were able to extract from these organisms an enzyme preparation capable of releasing the sapogenin. They considered that this was a broad-spectrum system and not specific to one saponin.

Ewart (1931) was of the opinion that saponins were detoxified in the liver, as animals with extensive liver damage were more readily affected by oral doses than were normal animals. He also suggested that prolonged treatment with some saponins could lead to liver damage. The work of Fieger (1918) showed that the sapogenin was excreted in the urine, indicating some absorption from the intestine after oral administration. In the case of quillaia, however, the degree of absorption appeared to be low. A lack of absorption from the gastro-intestinal tract was confirmed by Erbring & Vogel (1963), working with horse-chestnut saponin, and by Newman, Kummerow & Scott (1958), who showed that daily oral doses of 0·2 g digitoxin caused no haemolysis in cats whereas a marked haemolysis occurred within 10 min of iv injection. Vogel & Marek (1962) reached a similar conclusion with a range of saponins, which they found were considerably less toxic after oral administration than after iv injection. A lack of absorption in the case of soya-bean saponin was confirmed by blood analysis in rats, mice and chickens following oral doses (Gestetner *et al.* 1968).

It has been reported that quillaia saponin causes an irritation of the gastro-intestinal tract in hens and dogs (Bäck, 1917), and Brune & Günther (1961) reported a marked diarrhoea in rats given diets containing pure saponins. The *Extra Pharmacopoeia* (1972) quotes the toxic effect of quillaia extract as a violent local irritation of the gastro-intestinal mucosa. This can be severe enough to allow absorption and the development of systemic signs such as haemolysis, liver damage, respiratory failure, bladder irritation, convulsions and coma. Vogel & Marek (1962) confirmed the irritancy of saponins using the mucosa of the rabbit eye. They found that the lowest concentration causing visible damage ranged with various saponins from 1 in 2300 to 1 in 10,000.

Leroy & Marbarger (1969) exposed hamsters to an atmosphere containing droplets of 0·5 or 1·0% solutions of quillaia saponin. After 60–90 days with 1 hr exposure daily there was hyperplasia of the bronchial epithelium and focal lesions consisting of giant cells and histiocytes containing haemosiderin and fat. It seems probable that this effect may be related to the irritant properties of the saponin.

In *in vitro* systems, saponins cause haemolysis, although the degree of activity varies considerably (Ewart, 1931). Oser (1966) showed that a concentration of 1·78 g quillaia extract/100 ml caused haemolysis of 50% of a sample of washed rat erythrocytes. In general this activity, most easily detected with washed erythrocytes, is reduced by the presence of serum and even more reduced by cholesterol, with which saponins form complexes (Solé, 1954). Because of this protective action of cholesterol, Ewart (1931) considers that haemolysis only occurs *in vivo* with relatively large doses of saponins, even after iv treatment. It has been suggested (Glauert, Dingle & Lucy, 1962) that haemolysis results from the complexing of saponin with the cholesterol of the erythrocyte membrane, causing a rearrangement of the cholesterol so as to leave pores in the membrane.

Diets containing quillaia saponin caused a reduced rate of body-weight gain in chickens and this was reversed by the addition of cholesterol to the diet (Peterson, 1950). This reversal was thought to be due to combination in the intestine rather than to high serum cholesterol levels. The ability of cholesterol to reverse the impairment of body-weight gain caused by quillaia saponin was disputed by Newman *et al.* (1958) who considered that the differences were due to changes in food intake. A dose-related reduction in the rate of body-weight gain was seen in rats fed on diets containing 0·5–2·0% quillaia saponin while a level of 3·0% was lethal (Coulson & Evans, 1960). There was no consistent evidence from their studies that this effect was prevented by cholesterol. Oser (1966) fed a diet containing 0·05% quillaia extract to rats for 12 wk with no effect on weight gain, food intake, haematology (including erythrocyte fragility), urine analysis, organ weights, serum chemistry or the incidence of histopathological findings.

It has been suggested that saponins have an anti-vitamin D property (Brune & Günther, 1961), as their simultaneous administration prevented the sparing effect of vitamin D in rats fed on a rachitogenic diet. However, the work of Coulson & Evans (1960) with quillaia saponin failed to demonstrate this effect. It has been reported that there is a reduction of the activity of the enzymes of the Krebs cycle in tissues exposed to saponins *in vitro* (Cheeke, 1971), and Shaw & Jackson (1957) found that clover extracts reduced the *in vitro* respiration of the rat diaphragm. They also found a reduction in spontaneous intestine motility and in erythrocyte cholinesterase activity. In view of this latter observation the suggestion of Ewart (1931) that saponins may lead to central nervous system damage is of interest.

The present paper gives the results of a short-term study of quillaia extract in rats, carried out as part of the BIBRA Safety Evaluation Programme. It is part of a series of studies including long-term work in rats and mice, which will be reported later.

EXPERIMENTAL

Materials. The sample of quillaia extract used in these studies was supplied by Food Industries Ltd., Birkenhead, Cheshire. It was a spray-dried aqueous extract of quillaia bark, prepared in such a manner that 100 parts by weight of bark yielded approximately 15 parts of extract. In addition, the sample contained 5% lactose added to the extract before drying. The extract was stated to contain less than 10% moisture and less than 10% ash (at 550°C).

Animals. Weanling rats of the CFE strain, obtained from an SPF breeding colony, were housed in an animal room maintained at 20 ± 1°C and a relative humidity of 40–50%. Spillers' Laboratory Small Animal Diet and water were available *ad lib.*

Experimental design and conduct. Groups of 15 male rats (130–175 g body weight) and 15 females (105–135 g body weight) were housed five in a cage and fed on diets containing 0 (control), 0·6, 2·0 or 4·0% quillaia extract for 13 wk. Groups of five male and five female rats from the same batch were fed on diets containing 0 (control), 2·0 or 4·0% quillaia extract for 2 or 6 wk.

The animals were weighed and the food intake was measured before the experimental diets were fed and then weekly throughout the study. At the end of the appropriate feeding period, the rats were killed by exsanguination under barbiturate anaesthesia and an autopsy was conducted, during which brain, pituitary, thyroid, heart, liver, spleen, stomach, small intestine, caecum, kidneys, adrenals and gonads were weighed. Samples of these organs and of oesophagus, colon, rectum, lung, lymph nodes, skeletal muscle, trachea, uterus, urinary bladder and pancreas were preserved in 10% buffered formalin. Paraffin-wax sections of the tissues from the animals fed for 13 wk on the diet containing 4·0% quillaia extract and from half the control animals were stained with haematoxylin and eosin for microscopic examination.

Blood collected at autopsy was examined for haemoglobin content, packed cell volume and counts of erythrocytes, reticulocytes, leucocytes and the different types of leucocytes. *In vitro* haemolysis was assessed at wk 6 using the blood of five animals of each sex from the control group and that fed 4% quillaia extract. At wk 13, five females from these two groups were examined. The blood was diluted 1 : 100 in 0·85, 0·60 or 0·40% buffered saline (Dacie & Lewis, 1968) or in distilled water. The haemoglobin liberated was estimated after 30 min at room temperature and the values in saline were expressed as a percentage of the values in distilled water (total haemolysis).

Serum collected at autopsy was examined for contents of urea, glucose, total protein, and albumin and for the activities of the glutamic–oxalacetic transaminase, glutamic–pyruvic transaminase and lactic dehydrogenase.

During the last week of the feeding period, urine was collected from all rats and examined for microscopic constituents and content of blood, bile and ketones. A concentration test was carried out involving measuring the specific gravity and volume of urine produced in a 6-hr period without water. At wk 6 and 13 this was extended to include similar measurements on urine produced in a 2-hr period immediately after a water load of 25 ml/kg and 16–20 hr after the water load. A count of the number of cells in the urine was made using the 6-hr collection.

RESULTS

No abnormalities of behaviour or condition were seen in the rats fed on the diets containing quillaia extract. Particularly it was noted that there was no diarrhoea or other sign of gastro-intestinal irritation. The rate of body-weight gain was reduced at all levels of feeding, although the weight gain of the treated rats over the whole experimental period did not differ significantly from that of the controls (Table 1). The greatest difference in weight gain occurred in the first few days of feeding, and at the highest level (4·0%) there was a weight loss over the first 24 hr. The differences between the body weights of control rats and those given 4·0% quillaia saponin were statistically significant up to day 78 in males but only for the first 2 wk in females. Food consumption was reduced throughout the study in both sexes at all dietary levels (Table 1) and the mean intakes over the whole experimental period were significantly less than controls in females given 2·0 or 4·0% quillaia saponin

Table 1. *Body weight and food and water intake of rats fed on diets containing 0–4·0% quillaia extract for 13 wk*

Dietary level (%)	Body weight (g) at day				Weight gain (g) at day 92	Food intake (g/rat/day) at day				Mean intake (g/rat/day)	Water intake (ml/rat/day) at day				Mean water intake (ml/rat/day)
	0†	29	57	92		1	29	57	92		2	31	59	94	
Males															
0	150	333	428	496	346	19·4	24·0	22·0	21·3	21·9	25·3	29·5	28·5	25·0	27·9
0·6	150	319	407	466	316	18·2	26·4	19·4	21·5	20·5	25·0	20·9	26·3	23·6	25·9
2·0	150	321	419	478	328	14·7	22·9	20·4	20·2	20·3	23·4	26·9	24·9	25·8	25·1*
4·0	150	306***	409*	457	307	9·4	22·3	20·4	20·2	20·0	24·9	26·5	25·2	23·8	23·0***
Females															
0	123	214	263	272	149	15·6	22·7	17·5	17·2	17·8	21·3	22·2	21·6	25·0	23·1
0·6	121	215	264	293	172	15·6	19·2	17·2	17·6	17·4	22·6	22·0	23·9	25·6	23·9
2·0	123	207	251	277	154	13·3	16·8	11·3	12·4	14·0**	20·3	22·4	24·6	28·0	23·4
4·0	122	206	254	281	159	6·4	15·5	15·1	16·6	15·3**	19·0	21·0	22·2	24·4	21·4

†First day of feeding.

Values are the means for groups of 15 rats for body weight, and for three cages of five rats for food intake and water consumption. The values marked with asterisks differ significantly (Student's t test for body weight and ranking method of White, 1952, for food intake and water consumption) from those of controls: $*P < 0·05$; $**P < 0·01$; $***P < 0·001$.

in the diet. The largest differences from the control values were seen in the early part of the study, particularly during the first 24 hr.

The water consumption (Table 1) was reduced in males at all levels of treatment, and the mean intake over the experimental period in the case of the two highest quillaia levels (2·0 and 4·0%) was less than the controls to a statistically significant degree. In the females, this effect was seen only at the highest level of feeding. Calculation of the levels of intake of saponin over the experimental period established a mean daily intake of 0·36, 1·18 and 2·47 g/kg in males and 0·44, 1·37 and 3·03 g/kg in females given dietary levels of 0·6, 2·0 and 4·0% respectively.

No adverse differences between treated and control rats were seen in the haematological examinations, the results of which at wk 13 are given in Table 2, and the erythrocytes from treated rats were no more susceptible to haemolysis in hypotonic salt solutions than were those of controls. There were no differences between treated and control rats in the results of the serum analyses or in the urinary cell counts and renal concentration tests (Table 3) and no abnormal constituents were found in the urine.

No abnormalities were seen at autopsy and the significant differences in organ weights were confined almost entirely to males and consisted of decreases in the weights of the liver, spleen, kidneys, adrenals and pituitary, in some cases only at wk 2 or 6. These changes were found in groups that had low average body weights, and when the organ weights were expressed relative to body weight, there were no differences from controls in the adrenal and pituitary weights. The relative liver weights of males given 2·0 or 4·0% quillaia saponin were significantly lower than those of the controls, although no similar effect was seen in females. The relative spleen weight was reduced in females given 4·0% quillaia saponin in the diet for 2 wk and relative kidney weight was reduced with the highest dietary level in males at wk 6 and in females at wk 13. The organ weights at wk 13 are given in Table 4. Some increases in relative organ weight were seen. The relative stomach weights of rats fed on the diet containing 4·0% quillaia saponin were increased

Table 2. *Haematological findings in rats fed on diets containing 0–4% quillaia extract for 13 wk*

Dietary level (%)	Hb (g/100 ml)	PCV (%)	RBC (10^6/mm^3)	Retics (% of RBC)	Leucocytes Total (10^3/mm^3)	Differential (%) N	E	L	M
				Males					
0	14·8	46	7·29	1·0	6·54	15	1	81	3
0·6	14·7	46	7·15	1·0	5·39	14	1	82	3
2·0	14·5	46	7·27	1·3	5·64	18	0	79	3
4·0	14·6	44	7·15	1·2	5·94	17	1	79	3
				Females					
0	14·0	46	6·67	0·9	5·64	18	1	79	2
0·6	13·8	45	6·26	1·0	4·41	15	1	81	3
2·0	13·8	45	6·52	1·0	4·87	15	1	81	3
4·0	13·7	45	6·65	0·7	4·37	14	1	83	2

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

Values are means for groups of 15 rats. Basophils were not present at more than 0·5% in any group and no inclusions were seen in the erythrocytes. No adverse effects of the treatment were found at wk 2 and 6.

Table 3. *Results of urinary cell counts and concentration tests in rats fed on diets containing 0–4% quillaia extract for 13 wk*

Dietary level (%)	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)	Cell count (10^3/hr)
				Males			
0	1·054	1·067	2·0	1·1	1·009	5·6	3·7
0·6	1·053	1·070	1·6	0·7	1·009	7·9	3·5
2·0	1·047	1·070	1·8	0·7	1·009	6·6	3·0
4·0	1·055	1·063	1·2	0·5	1·013	5·2	3·3
				Females			
0	1·062	1·067	1·2	0·6	1·007	5·6	2·8
0·6	1·065	1·060	0·8	0·7	1·011	3·6	2·9
2·0	1·060	1·059	0·9	0·7	1·007	4·7	3·6
4·0	1·067	1·061	0·9	0·6	1·009	4·4	4·7

Values are means for groups of 12 rats. No differences between treated and control animals were found at wk 2 and 6.

throughout the study and at wk 13 the same effect was seen in males fed on the 2·0% diet. An increase in relative testis weight was seen at wk 2 at the highest dietary level.

The only histopathological changes seen were slight renal tubular dilatation and lymphocyte cuffing of the bronchi. The incidence of these findings was greater in the control rats than in those fed for 13 wk on the diet containing 4·0% quillaia saponin.

DISCUSSION

The reduced body-weight gain seen in this study is in keeping with results previously reported for chickens (Newman *et al.* 1958; Peterson 1950) and rats (Coulson & Evans, 1960). In the present study, the reduced rate of body-weight gain was transitory; it was most marked in the first few days of feeding and its persistence was dose related. There were parallel changes in the intakes of food and water. Such a reduction in food intake and body-weight gain occurring in the early stages of a study with subsequent recovery suggests the intake of an unpalatable diet rather than a toxic effect. It is likely that the reduction in water intake is also related to the reduced food consumption since such reductions have been seen in starved rats (Cizek & Nocenti, 1965) and in rats fed reduced quantities of food (Strominger, 1947).

The lack of any effect on the haematological parameters and the normal osmotic fragility of the erythrocytes extends the work of Oser (1966) to cover higher dosage levels.

The decreases in organ weight are difficult to interpret as they were seen in only one sex and were not associated with any histopathological change. However, particularly in the case of the liver, these reductions were seen consistently throughout the study. They cannot be accounted for solely in terms of differences of body weight, as similar changes were evident in the relative organ weights. Thus, until evidence to the contrary is produced, the dietary levels producing changes in both absolute and relative organ weights must be regarded as having a toxic effect.

Table 4. *Mean organ weights of rats fed on diets containing 0–4% quillaia extract for 13 wk*

Sex and dietary level (%)	Organ weights												Body weight (g)
	Brain	Heart	Liver	Spleen	Stomach	Small intestine	Caecum	Kidneys	Adrenals†	Gonads†	Pituitary†	Thyroid†	
						Weight (g)							
Male													
0	1·90	1·37	12·39	0·79	1·65	8·57	0·99	2·86	65	3·87	10·3	19	468
0·6	1·94	1·32	11·21*	0·83	1·65	8·09	1·08	2·67	58	3·74	11·7	23	443
2·0	1·93	1·32	10·86**	0·82	1·76	8·00	1·10	2·28*	64	3·70	9·8	15	453
4·0	1·88	1·29	10·57***	0·82	1·75	7·97	1·07	2·55*	56*	3·66	10·8	20	438
Female													
0	1·81	0·92	6·51	0·60	1·29	6·25	0·82	1·69	69	101	12·6	16	281
0·6	1·80	0·90	6·32	0·61	1·27	6·32	0·82	1·65	66	99	13·4	19	282
2·0	1·83	0·86	6·29	0·58	1·30	6·21	0·84	1·59	67	99	11·7	19	270
4·0	1·77	0·87	6·68	0·58	1·39	6·17	0·89	1·55	58	104	11·9	14	271
						Relative weight (g/100 g body weight)							
Male													
0	0·41	0·29	2·64	0·17	0·35	1·83	0·21	0·61	14	0·82	2·2	4·1	
0·6	0·44	0·30	2·53	0·19	0·37	1·83	0·24	0·60	13	0·85	2·6	5·1*	
2·0	0·43	0·29	2·40***	0·18	0·39**	1·77	0·24	0·57*	14	0·82	2·2	3·2	
4·0	0·43*	0·30	2·41***	0·19	0·40**	1·82	0·24	0·58	13	0·84	2·5	4·5	
Female													
0	0·65	0·33	2·32	0·21	0·46	2·24	0·29	0·60	25	36	4·5	5·8	
0·6	0·64	0·32	2·24	0·22	0·45	2·24	0·29	0·59	23	35	4·7	6·9	
2·0	0·67	0·32	2·32	0·21	0·48	2·30	0·31	0·58	25	37	4·3	7·0*	
4·0	0·65	0·32	2·45	0·21	0·51**	2·28	0·33*	0·57*	20	39	4·4	5·0	

†Values for these organs are expressed in mg and mg/100 g body weight.
‡Values for female gonads are expressed in mg and mg/100 g body weight.
Values are means for groups of 15 rats. Those marked with asterisks differ significantly (Student's t test) from those of controls: $*P < 0{\cdot}05$; $**P < 0{\cdot}01$; $***P < 0{\cdot}001$.

Similarly the increased stomach weight seen at the two higher dosage levels must be regarded as an effect of the saponin. These increases may be due to a local irritant effect, and as such, are likely to depend on the concentration present in the diet rather than on the total amount consumed. As the maximum concentration used in human food is likely to be 200 ppm (Food Additives and Contaminants Committee, 1970) and this enlargement of the stomach was seen only with concentrations of 20,000 ppm or above, this is unlikely to represent a hazard for man. Although this increase in stomach weight may be associated with an irritant effect, no signs, such as diarrhoea, were seen during the study and irritation was not confirmed by the findings at autopsy or in the histopathological examination.

On the basis of the results of this study, the no-untoward-effect level for quillaia extract is 0·6% of the diet of rats, a level equivalent to an intake of approximately 400 mg/kg/day. The lowest level at which any effects were seen was 2·0%, equivalent to approximately 1200 mg/kg/day, so the true no-effect level lies somewhere between these two figures. This point may be settled when the results of the long-term studies are available.

REFERENCES

Bäck, H. (1917). Beiträge zur Kenntnis der Ausscheidung der Saponine durch den Kot. *Biochem. Z.* **86**, 223.

Brune, H. u. Günther, K. (1961). Zur Frage der Wirksamkeit der Saponine als Antivitamin D. *Z. ErnährWiss.* **2**, 45.

Cheeke, P. R. (1971). Nutritional and physiological implications of saponins: A review. *Can. J. Anim. Sci.* **51**, 621.

Cizek, L. J. & Nocenti, M. R. (1965). Relationship between water and food ingestion in the rat. *Am. J. Physiol.* **208**, 614.

Coulson, C. B. (1958). Saponins. I.—Triterpenoid saponins from lucerne and other species. *J. Sci. Fd Agric.* **9**, 281.

Coulson, C. B. & Evans, R. A. (1960). The effect of saponin, sterols and linoleic acid on the weight increase of growing rats. *Br. J. Nutr.* **14**, 121.

Dacie, J. V. & Lewis, S. M. (1968). *Practical Haematology*. 4th ed. J. & A. Churchill Ltd., London.

Erbring, H. u. Vogel, G. (1963). Die Bedeutung physikalischer Zustandsänderungen für die enterale Resorption schwer resorbierbarer Saponine. *Arzneimittel-Forsch.* **13**, 467.

Ewart, A. J. (1931). The poisonous action of ingested saponins. *Council Sci. industr. Res. (Australia) Bull.* no. 50.

Extra Pharmacopoeia (1972). 26th ed. Edited by N. W. Blacow. p. 393. The Pharmaceutical Press, London.

Fieger, J. (1918). Über die Ausscheidung von Saponinen durch den Harn und ihre Wirkung auf das Blut nach innerlicher Darreichung. *Biochem. Z.* **86**, 244.

Food Additives and Contaminants Committee (1970). Report on the Review of the Emulsifiers and Stabilisers in Food Regulations 1962. HMSO, London.

Food Additives and Contaminants Committee (1972). Supplementary Report on the Review of the Emulsifiers and Stabilisers in Food Regulations 1962. HMSO, London.

Gestetner, B., Birk, Y. & Tencer, Y. (1968). Soybean saponins. Fate of ingested soybean saponins and the physiological aspect of their hemolytic activity. *J. agric. Fd Chem.* **16**, 1031.

Glauert, Audrey M., Dingle, J. T. & Lucy, J. A. (1962). Action of saponin on biological cell membranes. *Nature, Lond.* **196**, 953.

Gutierrez, J. & Davis, R. E. (1962). Isolation of saponin-digesting bacteria from the rumen of bloating cattle on Ladino clover pasture. *J. Anim. Sci.* **21**, 819.

Jackson, H. D. & Shaw, R. A. (1959). Chemical and biological properties of a respiratory inhibitor from alfalfa saponins. *Archs Biochem. Biophys.* **84**, 411.

Leroy, E. P. & Marbarger, J. P. (1969). Evolution of lesions in hamster's lung caused by inhaled aerosols of triterpenoid saponins from Quillaja saponaria. *Am. J. Path.* **55**, 41a.

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

Newman, H. A. I., Kummerow, F. A. & Scott, H. M. (1958). Dietary saponin, a factor which may reduce liver and serum cholesterol levels. *Poult. Sci.* **37** (1), 42.

Oser, B. L. (1966). An evaluation of *Yucca mohavensis* as a source of food grade saponin. *Fd Cosmet. Toxicol.* **4**, 57.

Peterson, D. W. (1950). Effect of sterols on the growth of chicks fed high alfalfa diets or a diet containing Quillaja saponin. *J. Nutr.* **42**, 597.

Shaw, R. A. & Jackson, H. D. (1957). Fractions from ladino clover which inhibit biological systems. *J. Anim. Sci.* **16**, 711.

Solé, A. (1954). Der Saponin-Hemm-Test. *Klin. Wschr.* **32,** 635.

Strominger, J. L. (1947). The relation between water intake and food intake in normal rats and in rats with hypothalamic hyperphagia. *Yale J. Biol. Med.* **19,** 279.

Vogel, G. u. Marek, Marie-Luise (1962). Zur Pharmakologie einiger Saponine. *Arzneimittel-Forsch.* **12,** 815.

White, C. (1952). The use of ranks in a test of significance for comparing two treatments. *Biometrics* **8,** 33.

Fd Cosmet. Toxicol. Vol. 12 pp. 651-663. Pergamon Press. 1974. Printed in Great Britain.

TWO-YEAR FEEDING AND MULTIGENERATION STUDIES IN RATS ON FIVE CHEMICALLY MODIFIED STARCHES*

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Abstract—Five chemically modified starches, acetylated distarch phosphate, acetylated diamylopectin phosphate, starch acetate, hydroxypropyl distarch glycerol and phosphated distarch phosphate, were fed to rats at dietary levels of 0 (control), 5, 10 and 30% for 2 yr and at one level, 10%, over three generations. In the 2-yr study, no adverse effects were observed on mortality, food intake, haematology, blood biochemistry or urine composition. Each of the modified starches examined, except the phosphated distarch phosphate, slightly reduced body weights at the 30% level and caused distinct caecal enlargement at 10 and 30%, but the microscopic structure of the caecal wall was normal. In comparison with the controls, the males fed the 30% level of any of the modified starches showed a slightly increased degree and incidence of focal hyperplasia of the renal papillary and pelvic epithelium, accompanied by calcified patches in the underlying tissue. The studies did not provide any indication of carcinogenicity. The multigeneration study showed no effect on fertility, on lactation performance or on embryonic or pre-weaning mortality. Extensive microscopic examination of the F_{3b}-generation rats failed to reveal any changes attributable to treatment. It was concluded that the feeding of each of the modified starches at dietary levels up to 30% for 2 yr and at a level of 10% over three generations did not result in any distinct effect of toxicological significance.

INTRODUCTION

Modified starches are prepared industrially from natural starches by chemical or physical treatment. During the past 25 yr, several of these products have been used to an increasing extent in a wide variety of food products to improve texture and consistency and to prevent separation of components. Chemical modification of starches usually involves the introduction of low levels of ether or ester groups. Sometimes modifying agents are used to connect neighbouring starch molecules and yield so-called "cross-linked" products.

Since the degree of substitution of modified food starches is usually low, it may be assumed that most of the anhydroglucose units in the starch are still released by enzymatic hydrolysis. This assumption is supported by results of *in vitro* digestibility studies with pancreatin (Janzen, 1969; Leegwater & Luten, 1971).

Biological determinations of calorific value in rats showed practically complete utilization of certain commercially modified food starches, but a very highly oxidized starch gave poor body-weight gains (Whistler & Belfort, 1961). In an *in vitro* study, it was found that the digestibility of hydroxypropyl starches decreased exponentially with increasing degree of substitution (Leegwater & Luten, 1971). A number of published and unpublished digestibility studies have recently been reviewed by Filer (1971).

*Studies carried out in co-operation with the Dutch starch industries: AVEBE G.A., CPC Nederland B.V. and Koninklijke Scholten-Honig N.V.

Only a few papers have been published on toxicity studies with modified starches. Baby pigs were fed on formula diets in which 30% of the dry matter consisted of either acid-treated starch (control), phosphated distarch phosphate or hydroxypropyl distarch glycerol. During a feeding period of 25 days the animals did not show growth depression, caecal enlargement or any other changes in blood chemistry or composition of the carcass and liver (Anderson, Filer, Fomon, Andersen, Jensen & Rogers, 1973).

A large number of unpublished reports on feeding studies with modified starches has been summarized by the Joint FAO/WHO Expert Committee on Food Additives (1972). From this compilation of data it appears that rats fed very high dietary levels of modified food starches in short-term studies usually exhibit slight growth depression, soft stools and hypertrophy of certain parts of the intestinal tract, notably the caecum. In order to obtain more information on the possible toxicological significance of the slight changes observed in rats fed modified starches for short periods of time, long-term feeding studies were conducted. The present report summarizes results obtained in rats fed five modified food starches in three separate 2-yr studies and in one multigeneration study.

EXPERIMENTAL

Starches. The five chemically modified starches examined, and the corresponding unmodified starting materials were:

Starch 1—unmodified potato starch, commercial name Prejel E 30, used as the control material for starches 2, 3, 4 and 5.
Starch 2—acetylated distarch phosphate, potato starch cross-linked with 0·02% phosphorus oxychloride and acetylated with 8% acetic anhydride (acetyl content 2·33%).
Starch 3—acetylated diamylopectin phosphate, amylopectin cross-linked with 1·2% phosphorus oxychloride and acetylated with 4·5% vinyl acetate (0·043% introduced phosphorus, acetyl content 1·6%), commercial name Paselli HVA-AC.
Starch 4—starch acetate, potato starch treated with 5% acetic anhydride (acetyl content 1·98%).
Starch 5—hydroxypropyl distarch glycerol, potato starch cross-linked with 0·1% epichlorohydrin and subsequently etherified with 5% propylene oxide, the degree of substitution with hydroxypropyl groups being 0·05 and 0·04 in two successive batches.
Starch 6—phosphated distarch phosphate, maize starch 'white milo', cross-linked with sodium trimetaphosphate up to 0·04% introduced phosphorus and esterified with sodium tripolyphosphate up to a total content of 0·35% bound phosphorus, commercial name Snow Flake 4832.
Starch 7—unmodified maize starch, used as the control material for starch 6.

All starches were precooked by drum-drying. Starches 1, 2, 4 and 5 were obtained from Koninklijke Scholten-Honig N.V., Foxhol, starch 3 from AVEBE G.A., Veendam, starches 6 and 7 from CPC Nederland B.V., Amsterdam.

Diets. In the 2-yr studies the modified starches were fed at three dietary levels, 5, 10 and 30%, and in the reproduction study one level, 10%, was fed. The starches were incorporated into basal diets at the expense of equal amounts of the corresponding precooked control starches. The percentage composition of the basal diet used in the long-term studies was as follows (the percentages in brackets being those used in the reproduction study where these differed): Fish meal, 8; meat scraps, 4; soybean-oil meal, 20; whole maize, 7 (16·5); whole wheat, 20 (30); grass meal, 3; brewer's yeast, 3; vitamin B preparation, 0·2;

choline chloride preparation, 0 (0·2); vitamin-ADEK preparation, 0·4 (0·6); steamed bone meal, 1; trace minerals in salt, 0·4; margarine, 3; control starch, 30 (10). Fresh batches of the diets were made every 2–3 wk and stored at room temperature.

Animals. Weanling male and female rats from the Institute's colony (Wistar-derived) were housed in groups of five, separated by sex, in screen-bottomed cages in well-ventilated animal rooms kept at 24–26°C.

Experimental design and conduct

Long-term study. The five modified starches were examined in three separate 2-yr studies. The diets were fed to groups of 30 male and 30 female rats. Food and tap-water were provided *ad lib.* Frequent observations were made of the condition and behaviour of the animals and of the appearance of the faeces. Individual body weights were recorded fortnightly during the first 12 wk and at 4-wk intervals thereafter. The food consumption was recorded at intervals during periods of 2 wk.

Haematological data, consisting of haemoglobin concentration, packed cell volume and counts of red blood cells and of total and differential white blood cells, were collected from ten males and ten females from each group at wk 13, 26, 52, 80 and 102. At wk 13, 26, 52, 81 and 103 blood was analysed for sugar and urea nitrogen with the Technicon AutoAnalyzer, using the Technicon standard procedure N-9[a] for glucose and N-10[a] for urea. Serum activities of glutamic–oxalacetic and glutamic–pyruvic transaminases and of alkaline phosphatase were measured terminally, as were total serum protein and serum albumin (modified after de Leeuw-Israel, Arp-Neefjes & Hollander, 1967). Pooled urine samples from ten rats from each group were examined for pH, sugar, protein, occult blood, ketones and microscopy of the sediment at several intervals.

After wk 104 all survivors were killed by decapitation and autopsied, and the major organs were weighed. Tissue samples were fixed in 10% phosphate-buffered formalin. A thorough autopsy was also performed on animals that died during the study or that were killed when moribund. Organs of these rats were not weighed, but tissues were preserved if autolysis was not too advanced. All tissues were embedded in paraffin, sectioned at 5 μm and stained with haematoxylin and eosin. The following tissues from each rat of the highest dose groups and of the three control groups were examined microscopically: heart, kidneys, liver, spleen, brain (three levels), testes, ovaries, adrenals, thymus, lung, trachea, salivary glands, oesophagus, stomach, intestinal tract (four levels), pancreas, urinary bladder, skeletal muscle, spinal cord, femoral nerve, skin, axillary and mesenteric lymph nodes, aorta, mammary gland, pituitary, thyroid, parathyroids, sternum with bone marrow, exorbital lachrymal glands and preputial gland, coagulating gland, uterus, prostate and seminal vesicle. Histological examination of tissues from the lower dose groups included the kidneys, urinary bladder, prostate and caecum of all rats, the adrenals of rats fed starches 2, 3, 4 and 5, and the thyroid and liver of rats fed starches 4 and 5. Special stains applied included periodic acid-Schiff, Azan, Gomori's method for reticulin fibres and von Kossa.

Reproduction and lactation study. Weanling rats from the stock colony were divided into seven groups each of ten males and 20 females to constitute the parent generation (F_0). The five test and two control diets as well as tap-water were constantly available. Individual body weights were recorded fortnightly until wk 12. At wk 12 and 20 all rats were mated in groups of five males and ten females on the same diet, to produce two successive litters (F_{1a} and F_{1b}, respectively). After a mating period of 3 wk the females were caged

individually until after the litters had been weaned. Records were made of the number of pups in each litter and the total weight of the litter at day 1, 10 and 20. Litters containing more than eight pups were randomly culled to eight on day 1. All young of the F_{1a} litters were discarded at weaning. When the F_{1b} litter reached weaning age, ten males and 20 females of each diet group were selected from as many litters as possible and maintained on the diet their parents had received. The procedure described for the F_0 generation was followed with the F_{1b} rats to produce rats of the F_{2a} and F_{2b} generations. This procedure was repeated with the F_{2b} offspring to produce the F_{3a} and F_{3b} litters.

After weaning their second litter, the mothers were killed. Each uterus was treated with ammonium sulphide solution for staining and the counting of implantation sites.

When the F_{3b} litters were weaned, ten males and ten females were selected from as many litters as possible in each diet group and continued on their diets for a period of 3 wk. Individual body weights were recorded weekly. During wk 4 all rats were killed and examined macroscopically for pathological changes. The weights of ten different organs were recorded and a wide range of organs and tissues was fixed in formalin. Detailed microscopic examination was carried out on all male and female rats of the test and control groups.

RESULTS

Long-term feeding studies

General condition, mortality and growth. No differences were observed between test animals and controls with respect to condition or behaviour. Diarrhoea did not occur, but some rats fed 30% of starch 5 produced soft stools. During the first 60 wk, deaths occurred only sporadically. Thereafter mortality increased rapidly in all groups but at no time were the numbers of deaths in a test group significantly higher than in the corresponding controls (Table 1).

Body weights did not show any marked growth retardation, although in the 30% group of each of the modified starches, except starch 6, the figures were consistently lower than in the corresponding control group (Table 2). At several stages the differences were statistically significant especially with starches 3 and 5. A slight reduction in growth rate occurred also with starch 5 at the 10% level in the first 3 months. The food intake figures of all groups were comparable at all stages. The food efficiencies, calculated over the period of rapid growth, showed a relatively low value only in males fed 30% of starch 5 (Table 2).

*Haematology**. The haematological indices revealed no significant abnormalities or treatment-related differences between the test and control groups at any of the stages. The terminal determinations showed a slight increase in leucocyte counts in females given 30% of starch 2 or 4 and a slight reduction in haemoglobin concentration in females receiving 30% of starch 5, but the values were within the normal ranges for the strain of rats used.

*Clinical chemistry**. Several statistically significant differences among test and control groups were observed in the results of the biochemical analyses of blood and serum, but there was no evidence of any dose-related response.

Urine analyses. Analysis of pooled urine samples collected at intervals from each dietary group did not reveal any effect of the modified starches on pH, sugar, protein, occult blood, ketones or sediment.

*Full data may be obtained from the authors.

Table 1. *Cumulative mortality in groups of 30 male and 30 female rats fed modified starches at dietary levels of 0–30% for 2 yr*

Starch in diet		Total no. of deaths in					
		Males at wk			Females at wk		
Type	Level (%)	72	96	104	72	96	104
1	(Control)	2	7	11	4	8	9
2	5	0	8	13	1	6	6
	10	4	12	18	0	3	7
	30	3	10	15	2	5	7
3	5	1	5	11	1	4	6
	10	3	11	16	1	4	7
	30	1	8	10	1	8	10
1	(Control)	3	11	11	0	2	4
4	5	2	11	12	0	3	4
	10	1	8	12	1	5	8
	30	4	14	19	0	2	4
5	5	5	11	14	1	4	6
	10	3	8	11	0	6	8
	30	2	8	14	1	7	11
7	(Control)	4	7	11	3	6	7
6	5	2	7	12	0	0*	9
	10	3	8	11	2	2	5
	30	3	9	15	1	4	7

The value marked with an asterisk differs significantly (chi-square test) from that of the corresponding controls: $*P < 0.05$.

Organ weights. The only dose-related changes in organ weight were found in the caecum (Table 3). There were increases in the relative weight of the filled and empty caecum of both sexes with the 30% dietary level of each of the modified starches. The differences were statistically significant except for the rats fed starch 6. This phenomenon occurred also with the 10% level in males fed starch 2, 3 or 4 and in females fed starch 5. Rats fed 30% of starch 6 showed higher caecal weights than did the corresponding controls, but the differences were not statistically significant. A few other organs showing slightly increased weights with the higher level of some of the starches revealed no treatment-related changes upon microscopic examination. and were, therefore, considered incidental findings.

Pathology. At autopsy, enlarged caeca were seen in a number of rats with the highest dosage level of the modified starches 2, 3, 4 and 5. Other changes observed during gross examination appeared to be randomly distributed. Histopathological findings included progressive nephrosis and hydronephrosis of the kidneys, bile-duct proliferation and hepatocyte vacuolization in the liver, pools of stagnant blood in the adrenals, morphological activation of the thyroid, chronic respiratory disease of the lung, focal myocarditis and myodegeneration of the heart, testicular atrophy and marked haemopoietic activity in the spleen. The lesions were randomly distributed among test and control animals, with the possible exception of a kidney abnormality which consisted mainly of focal hyperplasia of the renal papillary and pelvic epithelium accompanied by calcified patches in the underlying tissues. The hyperplastic and calcified tissues often protruded into the renal pelvis and were localized most often in the papilla near the junction of the papillary and pelvic epithelium. The lesion occurred to a slight or moderate degree in both sexes in most of the groups including the controls. Its incidence in the various groups was low (maximally 17%) but

Table 2. *Mean body weights and food efficiency of rats fed modified starches at 0–30% of the diet for 2 yr*

Starch in diet		Body weight (g) at wk						Food efficiency† at wk 1–4
Type	Level (%)	0	4	28	52	76	104	
				Males				
1	(Control)	75	191	378	437	481	488	0·31
2	5	75	190	382	427	459	491	0·32
	10	75	189	368	423	457	445*	0·32
	30	75	187	367	426	456	450	0·31
3	5	75	189	379	437	472	463	0·32
	10	75	195	382	430	468	433*	0·32
	30	75	182	358*	409*	440**	431**	0·30
				Females				
1	(Control)	69	132	223	248	285	308	0·22
2	5	69	132	224	256	287	309	0·22
	10	69	132	220	244	274	293	0·22
	30	69	132	222	248	277	282*	0·21
3	5	69	131	220	246	278	306	0·22
	10	69	130	222	245	276	308	0·22
	30	69	130	219	241	262*	273**	0·22
				Males				
1	(Control)	89	200	386	451	493	486	0·30
4	5	89	193	384	439	481	466	0·29
	10	89	196	382	439	464	468	0·30
	30	88	194	382	430	456*	470	0·28
5	5	89	192	387	442	481	481	0·28
	10	89	187**	378	431	467	501	0·27
	30	89	180***	367*	419**	451**	465	0·24
				Females				
1	(Control)	79	136	228	256	295	322	0·19
4	5	79	135	222	250	285	297	0·20
	10	79	133	220	250	284	309	0·18
	30	79	134	222	251	280	298	0·19
5	5	79	132	225	252	292	318	0·18
	10	79	129*	221	252	281	319	0·17
	30	79	128**	220	243*	269**	285*	0·17
				Males				
7	(Control)	51	192	386	427	467	416	0·37
6	5	51	185	378	420	466	445	0·37
	10	51	187	388	421	449	449	0·37
	30	51	184	394	425	457	446	0·37
				Females				
7	(Control)	50	136	235	262	301	318	0·29
6	5	50	134	227	256	292	301	0·29
	10	50	133	225	252	286	312	0·28
	30	50	137	230	260	297	303	0·29

†Food efficiency = weight gain (g)/food intake (g).

Body weights are the means for groups initially comprising 30 rats. Those marked with asterisks differ significantly (Student's t test) from the corresponding controls: $*P < 0·05$; $**P < 0·01$; $***P < 0·001$.

was always highest in males given the top dose level of each of the modified starches. Histopathological examination of the caecal wall revealed no indication of any morphological changes induced by the feeding of the test materials.

The type and incidence of the tumours observed are presented in Table 4. All neoplasms occurred in a random manner with no apparent relationship between number, site or type of tumour and the feeding of the various modified starches.

Reproduction and lactation study

The body weights of the rats did not differ among the groups in any of the successive generations and no treatment-related differences in mortality were observed between the test groups and controls. The data concerning reproductive performance are summarized in Table 5. Fertility remained excellent in all groups throughout the study. The growth rate of the pups in all groups given modified starches was comparable with that in the controls, and mortality *in utero* (resorption quotient) and preweaning mortality were low in all groups. The rats from the F_{3b} generation, sacrificed 3–4 wk after weaning, showed no gross or histological changes attributable to the feeding of the modified starches.

DISCUSSION

None of the modified starches examined in the present studies affected general condition, behaviour or survival, haematological or biochemical factors, urine composition or reproductive performance. The body weights of the rats fed 30% of any of the modified starches, except starch 6, were generally slightly lower than those of controls, but histological examination failed to reveal any distinct treatment-related changes. However, focal hyperplasia of the renal papillary and pelvic epithelium accompanied by calcification of the underlying tissue occurred to a slightly higher degree and frequency in males fed 30% of the test starches than in controls. A similar pathological process of unknown aetiology has been described recently in Manor Farms SPF rats (Bokelman, Bagdon, Mattis & Stoner, 1971), and in Sprague-Dawley rats (Magnusson & Ramsay, 1971). Because this renal change occurred only sporadically in the present study and, moreover, because there was neither a distinct relationship with the feeding level nor with the type of modification, its toxicological significance is doubtful.

The most marked alteration induced by the feeding of each of the modified starches, except starch 6, consisted of an increase in the size and weight of the caecum. Enlargement of the caecum and of other parts of the large intestine is known to occur in rats fed a variety of native carbohydrates (Fischer, 1957; Reussner, Andros & Thiessen, 1963) or modified starches (Joint FAO/WHO Expert Committee on Food Additives, 1972). Since caecal enlargement occurs soon after initiation of the feeding of the causative product (Fischer, 1957) and since the phenomenon was still present at the termination of the 2-yr feeding period, the particular intestinal condition was clearly present for the major part of the life-span of the animals. The fact that even the prolonged existence of this condition did not result in any relevant microscopic changes in the intestinal tract suggests that caecal enlargement is of little, if any, toxicological significance.

Although its aetiology is not known, caecal enlargement generally occurs with incompletely digestible carbohydrates and is often associated with soft stools and diarrhoea. The modified starches examined in the present studies also showed some resistance to enzymatic hydrolysis *in vitro*. The rate of digestion by pancreatin was found to be slightly retarded, and the digestibilities by pancreatin in combination with porcine intestinal mucosa varied between 81 and 95% of those of the corresponding starting materials (Leegwater, 1971). These findings, together with the absence of distinct signs of toxicity, indicate that the distension of certain parts of the large intestine is a physiological response to the presence of products resulting from incomplete digestion. The rapid disappearance of the condition after removal of the causative agent from the diet (de Groot, 1971) supports the assumption that caecal enlargement is an adaptive rather than a pathological condition. Recently

Table 3. *Relative organ weights of rats fed modified starches at dietary levels of 0–30% for 2 yr*

Starch in diet		Terminal body weight	No. of survivors	Relative organ weight (g organ weight/100 g body weight)								Caecum	
Type	Level (%)			Heart	Kidneys	Liver	Spleen	Brain	Gonads	Thyroid	Adrenals	Filled	Empty
						Males							
1	(Control)	491	19	0·345	0·63	3·08	0·160	0·39	0·68	0·0057	0·0107	0·95	0·24
2	5	496	16	0·340	0·71	3·02	0·162	0·41	0·61	0·0055	0·0105	0·95	0·27
	10	447*	12	0·355	0·70*	3·35	0·202	0·44*	0·78	0·0055	0·0111	1·09*	0·31***
	30	445*	14	0·344	0·68	3·18	0·149	0·43*	0·68	0·0063	0·0120	1·28*	0·33***
3	5	448	17	0·336	0·71*	3·12	0·139	0·45*	0·68	0·0052	0·0132	1·05	0·28
	10	437*	13	0·384*	0·74***	3·34*	0·154	0·46*	0·65	0·0063	0·0134	1·19***	0·32**
	30	422***	20	0·357	0·68	3·23	0·155	0·46*	0·72	0·0065	0·0134	1·14*	0·33***
						Females							
1	(Control)	307	19	0·377	0·67	3·55	0·199	0·60	0·018	0·0077	0·0163	1·08	0·31
2	5	309	23	0·364	0·63	3·17	0·188	0·59	0·021	0·0074	0·0167	1·10	0·35
	10	296	19	0·363	0·65	3·08*	0·193	0·63	0·018	0·0079	0·0185	1·00	0·32
	30	284	21	0·392	0·71	3·31	0·227	0·64	0·018	0·0078	0·0225**	1·35*	0·38*
3	5	311	23	0·376	0·65	3·26	0·256	0·59	0·018	0·0063	0·0183	1·09	0·30
	10	305	21	0·362	0·68	3·29	0·186	0·59	0·018	0·0079	0·0167	1·09	0·30
	30	271*	19	0·378	0·71	3·11	0·204	0·66	0·019	0·0074	0·0202	1·25	0·37

						Males
1	(Control)	483	19	0·355	0·68	3·23
4	5	470	16	0·361	0·66	3·17
	10	470	17	0·358	0·68	3·21
	30	465	11	0·377	0·67	3·20
5	5	472	16	0·348	0·64	3·15
	10	504	18	0·349	0·63	2·97*
	30	453	16	0·353	0·71	3·47
						Females
1	(Control)	314	23	0·369	0·63	3·02
4	5	294	25	0·366	0·64	2·99
	10	297	20	0·380	0·65	3·10
	30	297	24	0·370	0·66	3·38*
5	5	311	23	0·364	0·62	3·11
	10	314	22	0·369	0·61	3·31
	30	283*	17	0·379	0·66	3·29*
						Males
7	(Control)	416	19	0·384	0·70	3·27
6	5	445	18	0·362	0·73	3·31
	10	450	19	0·384	0·75	3·23
	30	447	15	0·383	0·75	3·20
						Females
7	(Control)	322	21	0·369	0·61	3·18
6	5	311	19	0·374	0·64	3·27
	10	316	23	0·365	0·65	3·17
	30	305	23	0·393	0·68**	3·47

Values and means for the numbers of animals indicated and those marked with asterisks
*$P < 0{\cdot}05$; **$P < 0{\cdot}01$; ***$P < 0{\cdot}001$.

0·171	0·42	0·60	0·0057	0·0130	1·00	0·27
0·161	0·42	0·63	0·0058	0·0111	1·00	0·26
0·162	0·42	0·60	0·0061	0·0134	1·22*	0·33*
0·185	0·43	0·62	0·0054	0·0125	1·22	0·37*
0·137**	0·42	0·68	0·0064	0·0127	1·11	0·29
0·153	0·40	0·59	0·0065	0·0101*	1·07	0·29
0·162	0·42	0·69	0·0067	0·0111	1·12	0·34*
0·206	0·59	0·019	0·0080	0·0159	1·02	0·31
0·191	0·63	0·018	0·0083	0·0171	1·02	0·35*
0·210	0·60	0·018	0·0080	0·0183	1·10	0·34
0·208	0·61	0·018	0·0068*	0·0203*	1·39**	0·38**
0·243	0·60	0·022	0·0069	0·0174	1·07	0·32
0·184	0·59	0·019	0·0068	0·0164	1·19	0·36**
0·193	0·63	0·020	0·0087	0·0185	1·40**	0·41***
0·165	0·47	0·65	0·0065	0·0139	1·23	0·28
0·169	0·44	0·73	0·0056	0·0116	1·15	0·29
0·147	0·44	0·63	0·0056	0·0128	1·16	0·29
0·136*	0·45	0·68	0·0070	0·0116	1·27	0·30
0·175	0·56	0·016	0·0075	0·0157	1·09	0·27
0·194	0·59	0·017	0·0069	0·0183	1·15	0·29
0·184	0·59	0·018	0·0079	0·0151	1·05	0·28
0·199**	0·60	0·017	0·0077	0·0177	1·17	0·30

differ significantly (Student's *t* test) from the corresponding controls:

Table 4. *Incidence and types of tumours in rats fed diets containing 30% modified or unmodified starch for 2 yr*

Site and type of tumour		Number of tumour-bearing rats															
		Males								Females							
	Starch in the diet...	1*	2	3	1*	4	5	7*	6	1*	2	3	1*	4	5	7*	6
	No. of rats examined...	30	28	28	23	27	30	28	28	29	30	28	30	30	26	29	29
	Total no. of rats with tumours...	26	25	24	21	24	19	21	23	21	25	18	23	17	20	25	22
Lung																	
Malignant lymphoreticular tumour		12	11	8	7	10	6	11	12	5	9	7	3	4	2	5	1
Adenoma		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
Adrenals																	
Phaeochromocytoma																	
Benign: small (hyperplasia?)		11	13	5	1	5	0	6	5	2	2	0	0	0	0	2	2
medium-sized		2	1	6	8	0	1	3	5	1	3	0	0	0	0	0	1
large		2	4	1	0	0	4	0	2	0	0	0	0	0	0	1	0
Malignant		4	0	1	1	1	0	0	0	0	0	0	0	0	0	0	0
Thyroid																	
Light-cell adenoma																	
Small (hyperplasia?)		6	4	3	1	1	1	0	2	5	4	6	4	2	1	4	4
Medium-sized		2	0	2	4	1	1	1	1	2	1	2	1	2	1	1	1
Large		1	0	3	1	0	0	1	1	1	2	1	2	0	1	2	0
Light-cell carcinoma		0	1	1	1	0	0	0	0	1	0	0	0	0	0	0	0
Sarcoma (?)		0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
Pituitary																	
Adenoma																	
Small (hyperplasia?)		1	1	2	1	1	1	6	4	3	8	2	3	1	0	3	2
Medium-sized		2	0	0	0	1	0	1	0	1	2	0	0	2	3	6	3
Large		1	1	0	1	2	4	0	0	3	2	3	1	2	1	1	2
Carcinoma		0	0	0	1	0	0	0	2	0	0	0	0	0	0	0	0

Site / tumour type	
Mammary glands	
Adenoma	0
Adenocarcinoma	0
Squamous-cell carcinoma	0
Fibroadenoma	0
Fibroma	0
Skin/subcutis	
Epidermoid carcinoma	0
Trichoepithelioma	0
Fibroma	1
Sarcoma	0
Abdomen	
Malignant lymphoreticular tumour	0
Sarcoma	0
Brain	
Meningioma	0
Thymus	
Malignant lymphoreticular tumour	0
Forestomach	
Papilloma	0
Liver	
Hepatoma	0
Pancreas	
Islet-cell tumour	1
Testes	
Interstitial-cell tumour	0
Ovaries	
Adenoma	
Uterus	
Polyp	
Adenocarcinoma	
Squamous-cell carcinoma	
Leukaemia	1
Total number of primary tumours...	47

*Unmodified control starch.

0	0	0	0	0	0	0	2	0	0	6	4	6	2	2
1	0	0	1	0	0	0	0	0	0	0	0	0	1	1
0	0	0	0	0	1	0	0	0	0	0	0	0	0	0
0	0	0	1	0	0	0	4	8	1	1	4	1	9	5
0	0	0	0	0	0	0	0	0	0	0	0	0	1	1
1	0	2	0	0	0	0	0	0	0	0	0	0	0	0
0	0	0	0	0	0	1	0	0	0	0	0	0	0	0
0	0	0	0	0	1	0	0	0	0	0	0	0	0	0
0	0	0	0	0	1	0	0	1	0	0	1	1	0	0
1	0	1	1	2	0	1	2	1	0	2	1	3	0	2
0	0	0	0	0	1	0	0	1	0	0	0	0	0	0
0	0	0	0	0	0	1	1	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0	1	0	0	0	0	0	0
0	0	0	0	0	0	0	1	0	1	0	0	0	0	0
0	0	0	0	1	0	0	0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
0	0	0	0	1	0	0								
							0	0	0	0	0	0	1	0
							1	3	5	0	2	1	5	7
							0	1	0	0	0	0	0	0
							0	0	0	0	0	0	0	1
1	5	0	2	2	0	0	2	0	0	2	2	3	1	2
40	38	30	27	24	33	37	37	49	28	25	27	24	45	38

Table 5. *Reproduction data for F_0–F_3 generation in rats fed modified starches at a dietary level of 10%*

Starch in diet†	Percentage of females with litter‡	Average no. of rats/litter at birth	Mean body weight (g) of young at day 1	10	20	Mortality (%) in young at day 10	20	Resorption quotients§
			F_0, first mating					
1	100	9·9	6·4	20·8	42·0	0·7	1·4	
2	100	10·4	6·4	20·2	40·9	0	0·6	
3	95	10·7	6·1	20·8	41·4	1·4	1·4	
4	90	10·9	5·9	19·8	40·4	2·1	2·1	
5	100	10·5	6·2	21·3	41·3	0·7	0·7	
6	100	10·5	6·4	20·7	42·2	4·6	4·6	
7	100	11·7	6·0	19·8	40·2	0·7	0·7	
			F_0, second mating					
1	100	10·8	6·2	20·9	41·9	0	0	1·09
2	100	11·7	6·3	20·4	40·7	1·2	1·2	1·01
3	100	11·3	6·2	19·7	39·9	0·6	0·6	0·96
4	95	11·8	5·9	19·4	39·3	0	0	1·14
5	100	11·1	6·5	21·1	43·0	0	0	0·99
6	100	11·2	6·3	21·0	42·9	0·7	0·7	1·08
7	95	12·6	5·8	20·5	41·0	1·4	1·4	1·00
			F_{1b}, first mating					
1	100	10·7	6·5	19·8	38·3	2·0	2·0	
2	100	9·2	6·6	20·4	40·8*	0·7	0·7	
3	100	9·7	6·3	19·8	37·8	1·9	1·9	
4	100	9·6	6·5	19·8	38·2	0	0	
5	95	11·2	6·2	19·8	38·5	0	0	
6	100	10·1	6·5	20·1	39·5	0	0	
7	100	9·9	6·4	19·5	38·8	0	0·7	
			F_{1b}, second mating					
1	100	10·6	6·6	21·3	42·7	0	0	1·10
2	95	11·2	6·6	21·2	42·2	0	0	1·06
3	95	11·3	6·3	20·9	41·4	0·7	0·7	1·08
4	100	10·1	6·3	21·2	42·3	1·4	1·4	1·09
5	100	12·1	6·4	20·1	40·5	3·1	3·8	1·04
6	100	10·6	6·4	21·0	41·4	2·6	2·6	1·07
7	100	10·3	6·3	20·9	41·9	1·4	1·4	1·13
			F_{2b}, first mating					
1	100	11·2	6·6	21·6	40·6	5·3	5·3	
2	90	9·9	7·2	22·8	44·7**	0	0	
3	100	10·1	6·7	21·7	41·1	0	0	
4	100	10·7	6·8	21·0	41·2	0	0·6	
5	95	9·5	6·5	20·9	40·9	0	0	
6	100	11·1	6·6	22·5	39·4	1·3	1·3	
7	95	11·1	6·4	21·5	41·1	1·3	1·3	
			F_{2b}, second mating					
1	100	10·9	6·3	21·2	42·2	1·4	7·2	1·06
2	100	11·0	6·7	23·9*	45·3*	0·7	1·4	1·07
3	100	11·2	6·4	22·1	42·2	0	0	1·06
4	100	9·5	6·7	23·1	43·7	2·0	2·0	1·05
5	100	10·1	6·4	21·6	41·4	0	0	1·04
6	90	10·1	6·4	22·0	42·3	0	0	1·04
7	90	10·5	6·4	22·8	43·3	0	0·8	1·08

†Groups fed diets containing unmodified starches 1 and 7 were used as controls for groups given starches 2, 3, 4 and 5 and starch 6, respectively.
‡No. of females mated in each group was 19 or 20.
§No. of implantation sites/no. of young born.
Values marked with asterisks differ significantly (Student's t test) from the corresponding controls: $^{*}P < 0.05$; $^{**}P < 0.01$.

it has been postulated that diet-induced caecal enlargement is due to osmotic processes (Leegwater, de Groot & van Kalmthout-Kuyper, 1974).

The results of the present studies, therefore, lead to the conclusion that starches of the kind and of the type and degree of chemical modification examined may be fed to rats at dietary levels up to 30% for 2 yr and at a level of 10% over three generations without inducing any distinct effects of toxicological significance.

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REFERENCES

Anderson, T. A., Filer, L. J., Jr., Fomon, S. J., Andersen, D. W., Jensen, R. L. & Rogers, R. R. (1973). Effect of waxy corn starch modification on growth, serum biochemical values and body composition of Pitman–Moore miniature pigs. *Fd Cosmet Toxicol.* **11**, 747.

Bokelman, D. L., Bagdon, W. J., Mattis, P. A. & Stoner, P. F. (1971). Strain-dependent renal toxicity of a nonsteroid antiinflammatory agent. *Toxic appl. Pharmac.* **19**, 111.

de Groot, A. P. (1971). Comparison of intestinal effects in rats induced by feeding either a chemically modified starch or lactose. Unpublished report No. R 3436 by Central Institute for Nutrition and Food Research (CIVO) TNO, Zeist.

de Leeuw-Israel, F. R., Arp-Neefjes, J. M. & Hollander, C. F. (1967). Quantitative determination of albumin in microlitre amount of rat serum; with a short note on serum albumin levels in ageing rats. *Exp. Gerontol.* **2**, 255.

Filer, L. J., Jr. (1971). Modified food starches for use in infant foods. *Nutr. Rev.* **29**, 55.

Fischer, Jessie E. (1957). Effects of feeding diets containing lactose, agar, cellulose, raw potato starch or arabinose on the dry weights of cleaned gastrointestinal tract organs in the rat. *Am. J. Physiol.* **188**, 550.

Janzen, C. J. (1969). Verdaulichkeit von Stärken und phosphatierten Stärken mittels Pankreatin. *Stärke* **9**, 231.

Joint FAO/WHO Expert Committee on Food Additives—Fifteenth Report (1972). Evaluation of Food Additives. Some Enzymes, Modified Starches, and Certain other Substances: Toxicological Evaluations and Specifications and a Review of the Technological Efficacy of some Antioxidants. *Tech. Rep. Ser. Wld Hlth Org.* **488.**

Leegwater, D. C. (1971). Preliminary study on the *in vitro* digestion of a number of chemically modified starches. Unpublished report No. R 3431 by Central Institute for Nutrition and Food Research (CIVO) TNO, Zeist.

Leegwater, D. C., de Groot, A. P. & van Kalmthout-Kuyper, Marina (1974). On the aetiology of caecal enlargement of rats on diets with chemically modified starches. *Fd Cosmet. Toxicol.* **12**, 687.

Leegwater, D. C. & Luten, J. B. (1971). A study on the *in vitro* digestibility of hydroxypropyl starches by pancreatin. *Stärke* **23**, 430.

Magnusson, G. & Ramsay, C. H. (1971). Urolithiasis in the rat. *Lab. Anim.* **5**. 153.

Reussner, G., Jr., Andros, J. & Thiessen, R., Jr. (1963). Studies on the utilization of various starches and sugars in the rat. *J. Nutr.* **80**, 291.

Whistler, R. L. & Belfort, A. M. (1961). Nutritional value of chemically modified corn starches. *Science, N.Y.* **133**, 1599.

Fd Cosmet. Toxicol. Vol. 12 pp. 665 669. Pergamon Press 1974. Printed in Great Britain

DEVELOPMENT AND REVERSIBILITY OF PANCREATIC ACINAR CELL CHANGES IN THE RAT PRODUCED BY TEBUTHIURON (1-(5-*tert*-BUTYL-1,3,4-THIADIAZOL-2-YL)-1,3-DIMETHYLUREA)

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Abstract—Vacuolization and a decrease in zymogen granules in the pancreatic acinar cells were observed in Sprague-Dawley rats fed a ration containing 2500 ppm tebuthiuron. These changes were rapidly induced and progressive, and upon withdrawal of the treatment were rapidly reversed. A depression of body-weight gain appeared to be directly related to the severity of the pancreatic changes. Structural changes in the pancreatic acinar cells, demonstrated by light and electron microscopy, were characterized by membrane-bound vacuoles containing dense membranes, by myeloid bodies and by a reduction in zymogen granules. These pancreatic changes were interpreted as being related to an interference with protein synthesis.

INTRODUCTION

Vacuolization of pancreatic acinar cells with a reduction in zymogen granules was reported by Todd, Gibson & Kehr (1974) as the only pathological evidence of toxicity in Wistar-derived rats fed rations containing 2500 ppm tebuthiuron (1-(5-*tert*-butyl-1,3,4-thiadiazol-2-yl)-1,3-dimethylurea) for 3 months. There was no evidence that this lesion was associated with inflammation or necrosis. The present study was initiated with three objectives, namely to establish the reproducibility of the lesion in another strain of rat, to follow the time sequence of the development of vacuolar changes in the pancreatic acinar cells by means of light and electron microscopy, and to determine the reversibility of the lesion.

EXPERIMENTAL

Material. The tebuthiuron used in this study was drawn from the same production lot as that used in previous studies by Todd *et al.* (1974).

Animals, diets and treatment. Following a few days of acclimatization, male Sprague-Dawley rats, weighing 105–146 g, were separated into two groups and caged individually in wire hanging cages with free access to food (Lilly mill rat ration) and water. One group of 40 rats was fed a ration containing 0·25% (2500 ppm) tebuthiuron, while the other group of 40 rats received untreated ration and served as controls. On test days 2, 4, 8, 15, 17, 19, 23 and 30, five rats from each group were autopsied, and pancreatic tissue was prepared for light and electron microscopic study. In order to determine the reversibility of the pancreatic changes, the group of rats receiving tebuthiuron on test days 1–15 were given untreated ration on days 16–30. Gross examinations were made of the body organs and samples of pancreatic tissue were placed in fixative. Surviving rats were weighed once weekly.

Pancreatic tissue for light microscopy was fixed in 10% buffered formalin, paraffin embedded, sectioned and stained with haematoxylin and eosin. Tissue processed for electron microscopy was immersed in veronal acetate–buffered 2% osmium tetroxide, dehydrated through graded alcohols and embedded in Epon 812. Ultrathin sections were placed directly on uncoated copper grids, stained with uranyl acetate and lead citrate, and examined with the electron microscope.

RESULTS

General condition, growth and mortality

All rats appeared normal and there were no deaths. A reduction in the rate of body-weight gain occurred during the period of tebuthiuron treatment. However, when treated rats were placed on the control ration (recovery period) their rate of weight gain paralleled that of the control rats (Fig. 1).

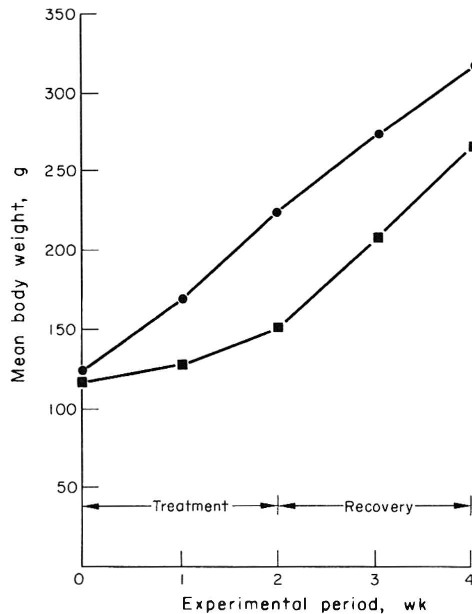


Fig. 1. Growth curves for male rats fed either the control ration (●) or a ration containing 2500 ppm tebuthiuron (■). A 2-wk treatment period was followed by a 2-wk recovery period. Note that the growth curve of treated rats was depressed during treatment but paralleled the curve for control rats during the recovery period.

Light-microscopic findings

Early focal vacuolization of the pancreatic acinar cells could be detected within 2–4 days in rats treated with 2500 ppm tebuthiuron (Fig. 2). This change was progressive and by 8 days there was extensive and diffuse vacuolization (Fig. 3; Table 1). Many vacuoles contained light-staining, foamy, amorphous material. With increased vacuolization there was a decrease in zymogen granules. No evidence of inflammation or necrosis was found. When treated rats were given rations containing no tebuthiuron, these pancreatic acinar

Table 1. *Incidence of microscopic changes in the pancreatic acinar cells of rats during treatment with a diet containing 2500 ppm tebuthiuron and during a subsequent recovery period*

	Incidence of changes*									
	Histological				Ultrastructural					
							Myeloid bodies†			
Test day	None	Focal vacuolization	Focal vacuolization and decreased zymogen	Diffuse vacuolization and decreased zymogen	Vacuole formation	Decreased zymogen	0	I	II	III
				Treatment period						
2	1	4	0	0	0	0	0	3	2	0
4	0	1	4	0	5	0	0	2	3	0
8	0	0	1	4	5	4	0	0	2	3
15	0	0	0	5	5	4	0	0	2	3
				Recovery period						
17	0	0	0	5	5	3	0	0	1	4
19	0	4	1	0	2	1	0	1	2	2
23	3	2	0	0	0	0	0	5	0	0
30	5	0	0	0	0	0	1	4	0	0

*No. of rats affected in each group of five. Two of five control rats were autopsied and examined by light and electron microscopy for pancreatic changes on each of the test days on which treated rats were killed and all were found to be normal.

†The degree of myeloid body formation in the cytoplasm was graded as follows: 0 (none), I (few), II (moderate), III (many).

changes were rapidly reversed. Following 8–15 days of recovery there were only a few focal cells with vacuoles, the remaining acinar cells appearing normal (Table 1).

Electron-microscopic findings

The normal ultrastructure of the rat pancreas has been adequately described by Ekholm, Zelander & Edlung (1962). The ultrastructure of pancreatic acinar cells from our control animals was in keeping with the description by these authors.

The earliest changes observed in rats treated with tebuthiuron for 48 hr involved the appearance of small electron-dense bodies in the cytoplasm of acinar cells (Table 1). Whorls of myeloid membranes in the cytoplasm were associated with the endoplasmic reticulum and Golgi apparatus, and later these were aggregated into dense myeloid bodies. The degree of myeloid-body formation in the cytoplasm was graded as 0 (none), I (few), II (moderate) and III (many), as shown in Table 1.

After 4 days treatment, a few small (1·5 μm) membrane-bound vacuoles appeared in the cytoplasm and some were filled with electron-dense membranes (Fig. 4). Several myeloid bodies were observed and, in a few cells, the Golgi apparatus was prominent. Distribution of zymogen granules appeared to be normal.

Treatment for 8 days produced vacuoles up to 3 μm in diameter in the cytoplasm. These membrane-bound vacuoles frequently contained electron-dense plaques and membranes. Some of the mitochondria were also undergoing focal changes and contained myelin figures. The Golgi apparatus increased in prominence in most cells and there was a decrease in zymogen granules.

The vacuoles increased in size and number by day 15 of treatment. Some vacuoles were 5 μm in diameter and many contained electron-dense membranes. A number of vacuoles

appeared to be closely associated with the Golgi complex (Fig. 5). Myeloid bodies were evident and zymogen granules were reduced in number (Table 1). Although rare, regions of focal degradation containing dense bodies and remnants of mitochondria were found (Fig. 5).

Rats treated for 15 days and then placed on the control diet for 48 hr showed a decrease in the number of vacuoles. The membrane-bound vacuoles contained many electron-dense membranes and a number of myeloid bodies were observed in the cytoplasm. Prozymogen granules were present.

Acinar cells of treated rats placed on the control diet for 4 days (test day 19) contained areas of cytoplasmic sequestration and products of degradation. These irregular, dense bodies contained amorphous electron-dense granules, myeloid bodies and vesicles. The laminated membranes of the Golgi complex were only moderately expanded and there was an increase in zymogen granules.

Acinar cells of treated rats allowed to recover for 8 or 15 days were essentially normal. A few myeloid bodies were present (Fig. 6), but this phase was characterized by the gradual disappearance of the cytoplasmic lesions and an increase in zymogen granules.

DISCUSSION

Vacuolization of pancreatic acinar cells with a decrease in zymogen granules was produced in Sprague-Dawley rats fed a ration containing 2500 ppm tebuthiuron. This pancreatic lesion was identical to that produced in Wistar-derived rats under similar experimental conditions (Todd *et al.* 1974).

Ultrastructural changes were evident by test day 2 and these were progressive through test day 15. The vacuoles were membrane-bound. and often contained electron-dense plaques and membranes. Myeloid bodies were present in the cytoplasm and in some cells the Golgi complex was expanded. The recovery period was characterized by a rapid disappearance of vacuoles, sequestration of cytoplasmic debris and products of degradation, a gradual diminution of myeloid bodies and an increase in zymogen granules.

The rate of body-weight gain was depressed during treatment, but during the recovery period the growth rate was comparable to that of the control rats. Progression of the pancreatic acinar changes was reflected in the growth curves. These findings are complementary and support the conclusion of a rapidly induced and rapidly reversed lesion.

Vacuolization of pancreatic acinar cells, similar to that produced by tebuthiuron, has been reported in rats fed moderate amounts of β-3-thienyl-DL-alanine in semi-synthetic diets lacking phenylalanine (Hruban, Swift & Wissler, 1962a), in puromycin-treated rats (Longnecker, Shinozuka & Farber, 1968) and in rabbits fed a protein-deficient diet (Lazarus & Volk, 1964). High doses of caerulein (a peptide from the Australian amphibian, *Hyla caerulea*) caused pancreatic vacuolization in dogs (Tardini, Anversa, Bordi, Bertaccini & Impicciatore, 1971). Volk, Wellmann & Lewitan (1966) reported irradiation-induced vacuolization in the pancreatic acinar cells of the dog.

Chemicals known to induce a loss of zymogen granules in the pancreatic acinar cells of the rat are 1-aminocyclopentane carboxylic acid (Chenard & Auger, 1968), β-3-thienyl-DL-alanine (Hruban, Swift & Wissler, 1962b), triparanol and diethanolamine (Hruban, Swift & Slesers, 1965) and β-3-furylalanine (Hruban, Swift, Dunn & Lewis, 1965). Depletion of zymogen granules has also been reported by Weisblum, Herman & Fitzgerald (1962) in protein-deprived rats and in lysine-deficient rats (Scott, 1966). Lazarus & Volk, (1964) observed a reduction in zymogen granules in protein-deficient rabbits and similar

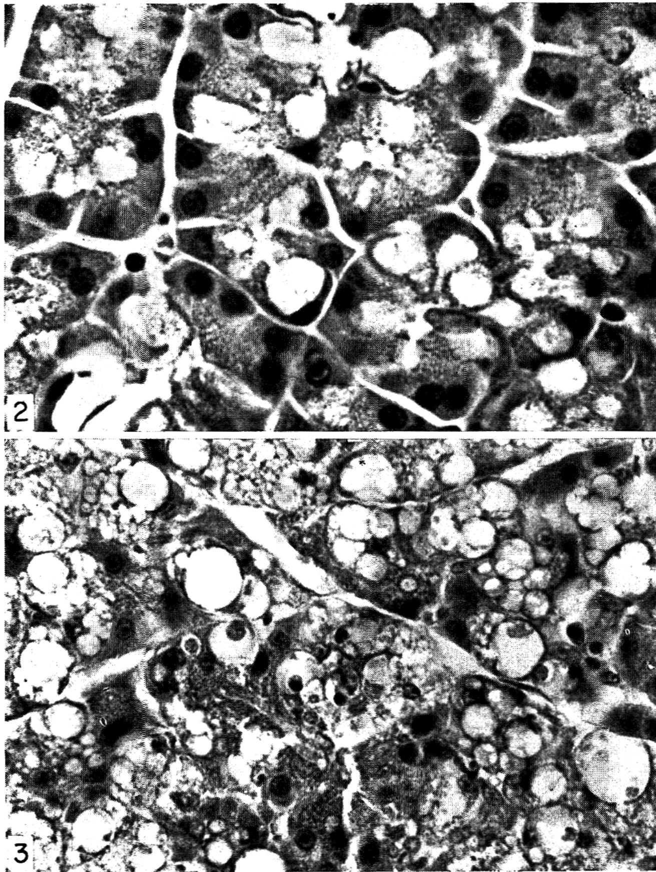
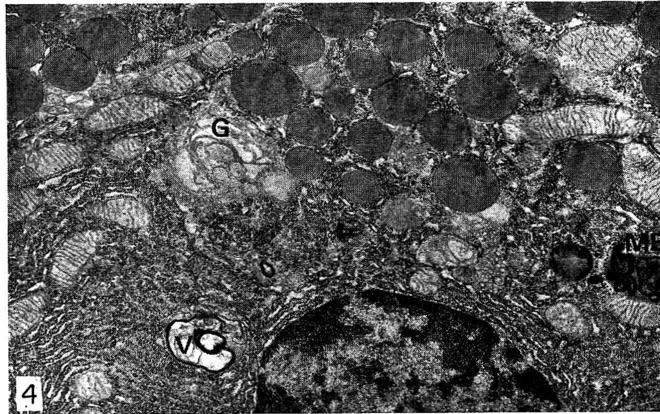
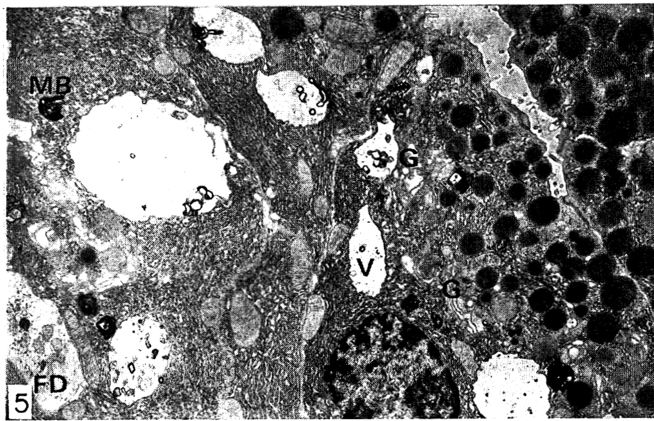


Fig. 2. Section of pancreatic acinar tissue from a rat given a diet containing 2500 ppm tebuthiuron for 4 days, showing vacuoles and zymogen granules in the cytoplasm. Haematoxylin and eosin. × 900.

Fig. 3. Section of pancreatic acinar tissue from a rat treated for 8 days with tebuthiuron, showing many vacuoles and a reduction in zymogen granules. Haematoxylin and eosin × 700.





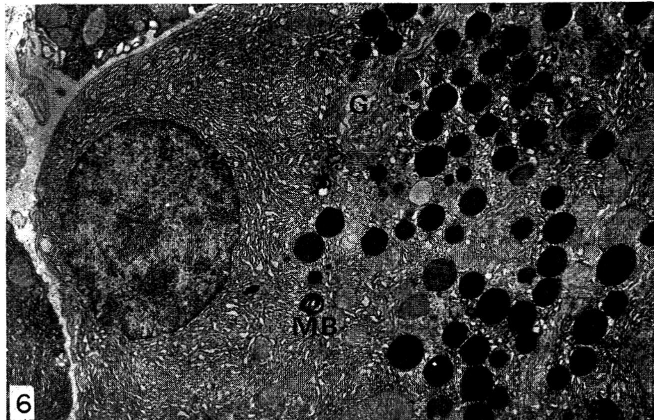


Fig. 4. Pancreatic acinar cell from a rat given 2500 ppm tebuthiuron for 4 days. A small vacuole filled with electron-dense membranes (V), a myeloid body (MB) and prominence of the Golgi apparatus (G) are depicted. Uranyl acetate and lead citrate × 11,375.

Fig. 5. Electron micrograph of pancreatic acinar cells of a rat given 2500 ppm tebuthiuron for 15 days. There are many vacuoles (V), some in close proximity to the Golgi complex (G). Myeloid bodies (MB) and a region of focal degradation (FD) are depicted. Uranyl acetate and lead citrate × 6600.

Fig. 6. Pancreatic acinar cell from a rat given 2500 ppm tebuthiuron for 15 days and allowed to recover for 8 days. The cell appears normal except for a few myeloid bodies (MB) in the field. G—Golgi complex. Uranyl acetate and lead citrate × 8250.

findings were reported by Volk *et al.* (1966) and by Wellmann, Volk & Lewitan (1966) in radiation studies with dogs.

The myeloid bodies observed in this study resembled those reported in the pancreatic acinar cells of rats treated with β-3-thienyl-DL-alanine (Hruban *et al.* 1962a) and in protein-deficient rabbits (Lazarus & Volk, 1964). These dense complex bodies have also been observed by Schoning, Anderson & Westfall (1972) in dogs treated with staphylococcal α-toxin and in radiation studies using dogs (Volk *et al.* 1966; Wellmann *et al.* 1966).

Pancreatic acinar cells produce, store and secrete abundant amounts of protein as digestive enzymes. The mechanisms underlying the production of vacuolization and the loss of zymogen granules by pancreatic acinar cells are unknown. These changes may be attributable to an interference with protein synthesis, inhibiting the production of normally-occurring enzymes or producing toxic abnormal proteins.

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REFERENCES

Chenard, J. & Auger, C. (1968). Cytoplasmic changes in pancreatic acinar cells of the rat caused by one-aminocyclopentane carboxylic acid (ACPC). A light and electron microscopic study. *Am. J. Path.* **52,** 825.

Ekholm, R., Zelander, T. & Edlund, Y. (1962). The ultrastructural organization of the rat exocrine pancreas: I. Acinar cells. *J. Ultrastruct. Res.* **7,** 61.

Hruban, Z., Swift, H., Dunn, F. W. & Lewis, D. E. (1965). Effect of β-3-furylalanine on the ultrastructure of the hepatocytes and pancreatic acinar cells. *Lab. Invest.* **14,** 70.

Hruban, Z., Swift, H. & Slesers, A. (1965). Effect of triparanol and diethanolamine on the fine structure of hepatocytes and pancreatic acinar cells. *Lab. Invest.* **14,** 1652.

Hruban, Z., Swift, H. & Wissler, R. W. (1962a). Analog-induced inclusions in pancreatic acinar cells. *J. Ultrastruct. Res.* **7,** 273.

Hruban, Z., Swift, H. & Wissler, R. W. (1962b). Effect of β-3-thienylalanine on the formation of zymogen granules of exocrine pancreas. *J. Ultrastruct. Res.* **7,** 359.

Lazarus, S. S. & Volk, B. W. (1964). Electron microscopy and histochemistry of rabbit pancreas in protein malnutrition (experimental kwashiorkor). *Am. J. Path.* **44,** 95.

Longnecker, D. S., Shinozuka, H. & Farber, E. (1968). Molecular pathology of in-vivo inhibition of protein synthesis. Electron microscopy of rat pancreatic acinar cells in puromycin-induced necrosis. *Am. J. Path.* **52,** 891.

Schoning, Polly, Anderson, N. V. & Westfall, Jane A. (1972). Effect of staphylococcal α-toxin on the fine structure of pancreatic acinar cells of dogs. *Am. J. Path.* **66,** 497.

Scott, E. B. (1966). Histopathology of amino acid deficiencies. VIII. Electron microscopy of the exocrine pancreas cells of lysine-deficient rats. *Archs Path.* **82,** 119.

Tardini, A., Anversa, P., Bordi, C., Bertaccini, G. & Impicciatore, Mariannina (1971). Ultrastructural and biochemical changes after marked caerulein stimulation of the exocrine pancreas in the dog. *Am. J. Path.* **62,** 35.

Todd, G. C., Gibson, W. R. & Kehr, C. C. (1974). Oral toxicity of tebuthiuron (1-(5-*tert*-butyl-1,3,4-thiadiazol-2-yl)-1,3-dimethylurea) in experimental animals. *Fd Cosmet. Toxicol.* **12,** 461.

Volk, B. W., Wellmann, K. F. & Lewitan, A. (1966). The effect of irradiation on the fine structure and enzymes of the dog pancreas. I. Short-term studies. *Am. J. Path.* **48,** 721.

Weisblum, B., Herman, L. & Fitzgerald, P. J. (1962). Changes in pancreatic acinar cells during protein deprivation. *J. Cell Biol.* **12,** 313.

Wellmann, K. F., Volk, B. W. & Lewitan, A. (1966). The effect of radiation on the fine structure and enzyme content of the dog pancreas. II. Long term studies. *Lab. Invest.* **15,** 1007.

Fd Cosmet. Toxicol. Vol. 12 pp. 671–679. Pergamon Press 1974. Printed in Great Britain.

STUDY ON THE CARCINOGENICITY OF LEAD ARSENATE AND SODIUM ARSENATE AND ON THE POSSIBLE SYNERGISTIC EFFECT OF DIETHYLNITROSAMINE*

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Abstract—In a lifetime (29-month) carcinogenicity study, rats were treated with lead arsenate ($PbHAsO_4$) or sodium arsenate ($Na_2HAsO_4 \cdot 7H_2O$) and the possibility that diethylnitrosamine (DENA) may exert a synergistic or additive action on the effects of these compounds was studied. Lead arsenate was fed in the diet at levels of 1850 or 463 ppm, while sodium arsenate was fed at a level of 416 ppm. Additional groups of rats were fed 463 ppm lead arsenate or 416 ppm sodium arsenate in combination with DENA given in an intubated dose of 5 μg/day on 5 days/wk. The rats receiving these diets were weaned by mothers receiving the same diet during lactation. Food intake levels (for the first 12 wk) and body weights were recorded, haematological studies were carried out after 12 months and complete gross and microscopic examinations were conducted at autopsy.

At a level of 1850 ppm, lead arsenate was toxic and caused intra- and extrahepatic bile-duct lesions. Intranuclear inclusions due to the ingested lead were present in the kidneys and liver, but only for the first 12 months. Other histopathological changes, commonly found in these rats, were equally divided between the groups. No differences were apparent either in the tumour incidence of the different groups or in the times at which tumours were detected. An adenoma in the renal cortex and a bile-duct carcinoma, both found in the group fed 1850 ppm lead arsenate, may have been indicative of a very weak carcinogenic action of this compound, but no definite conclusion can be drawn from these data. Sodium arsenate showed no carcinogenic action at the dose level used in this study. No additive or synergistic effect could be attributed to DENA, which also failed to induce tumours at the low dose level used, suggesting the possible existence of a no-effect level for this carcinogen.

INTRODUCTION

Epidemiological studies have indicated that some elements or their compounds may be carcinogenic to man. For chromium and nickel, the indications are clear and have been supported by studies in animals. For arsenic, however, a discrepancy exists between the evidence of epidemiological studies in man and the results of experiments in animals (International Agency for Research on Cancer, 1973). There is strong circumstantial evidence that arsenic and arsenic compounds can induce both skin and lung tumours in man (Lee & Fraumeni, 1969; Tseng, Chu, How, Fong, Lin & Yeh, 1968), but hitherto, experimental studies on laboratory animals have produced no clear evidence of the carcinogenic action of arsenic and its compounds (Frost, 1967; Miller & Miller, 1965; Neubauer, 1947; Vallee, Ulmer & Wacker 1960).

Lead has been shown to be carcinogenic in animals. In the form of basic lead acetate, it induces kidney tumours in rodents (Boyland, Dukes, Grover & Mitchley, 1962; Van

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Esch & Kroes, 1969; Van Esch, Van Genderen & Vink, 1962). Both lead and arsenic occur naturally in the environment, and in addition, are used extensively in the form of their compounds, such as tetraethyllead, used as an antiknock agent in motor fuel, and lead arsenate, used in agriculture. A study of the potential carcinogenicity of lead and arsenic in rats was therefore undertaken.

Since arsenic has been suspected of provoking lung tumours as well as tumours of the skin in man, it seemed of interest also to conduct a lifetime study in rats on the combined effects of arsenic and the lung carcinogen, diethylnitrosamine. In view of the conditions to which man may be exposed, the combined effect of lead compounds and diethylnitrosamine (DENA), with the possibility of a synergistic or additive effect between this metal and the nitrosamine in the induction of renal or other tumours, was also of importance. A lifetime experiment in rats was therefore initiated, to investigate whether sodium arsenate or lead arsenate is a carcinogen and whether any synergistic or additive action exists between DENA and lead and/or arsenic.

EXPERIMENTAL

Animals and treatment. Male and female SPF-derived Wistar rats were obtained from the Central Institute for the Breeding of Laboratory Animals TNO, Zeist, The Netherlands. The experiment was started with 200 females and 100 males, which received the normal standard commercial diet (Muracon, Trouw en Co., Putten, The Netherlands) and were litter-mate divided into four breeding-groups. From day 13 of pregnancy the females were fed the test diet to be fed later to their young for the carcinogenicity experiment. The breeding results were not satisfactory, however, and a second breeding experiment was started in which the experimental diet was fed to the dams only after the birth of their litters. After weaning, the young animals were divided into seven groups and started on the treatments indicated in Table 1. As the number of litters was limited, the age of the animals, and therefore their body weights, varied considerably at the beginning of the experiment. Insufficient animals were available in the group to be given 463 ppm lead

Table 1. *Design of study involving treatment of rats with lead arsenate or sodium arsenate and/or DENA for up to 29 months*

	Treatment				No. of animals/group	
	In diet		By intubation*			
Group no.	Lead arsenate (ppm)	Sodium arsenate (ppm)	DENA μg/day	Water (ml)	Males	Females
1a	—	—	—	0·3	50	60
1b	—	—	5	0·3	50	60
2	1850†	—	—	—	29	19
3a‡	463§	—	—	0·3	40	40
3b	463	—	5	0·3	40	40
4a	—	416§	—	0·3	40	40
4b	—	416	5	0·3	40	40

DENA = Diethylnitrosamine

*Oesophageal intubation, 5 times/wk.
†Equivalent to about 400 ppm arsenic.
‡This group was started on test at a later date.
§Equivalent to about 100 ppm arsenic.

arsenate and therefore a new breeding experiment was started in the manner described above. This group was therefore started later, without an additional control group (Table 1). Because the number of litters was sufficient in this group, the body weights at the start of the experiment were more uniform.

The animals were housed in wire cages, with two animals in each cage. They received the commercial diet, with or without added test compounds, and water *ad lib.* The rats treated orally with DENA were housed in a separate but similar animal room.

Materials. Technical lead arsenate ($PbHAsO_4$) was obtained from Mechema Ltd. (Port Talbot, Wales), as a purified white powder, giving the following results on analysis: lead, 64·6% PbO (approximately 60% Pb); arsenic, 32·0% As_2O_5 (approximately 20·9% As); water-soluble arsenic, 0·06% As_2O_5; acidity, 0·15% HNO_3. Sodium arsenate, from Merck AG, Darmstadt, Germany, consisted of white crystals with the following specification: $Na_2HAsO_4 \cdot 7H_2O$, min. 98·5%; water-insoluble material, max 0·005%; chlorine, max 0·002%; sulphate, max 0·002%; nitrate, max 0·002%; lead, max 0·00008%; iron, max 0·00003%; arsenic salts (As_2O_3), max 0·002%. DENA ($CH_3 \cdot CH_2 \cdot N(CH_3 \cdot CH_2) \cdot NO$) received from Schuchardt GmbH, München, Germany, was a liquid with b.p. 63–64°C.

Conduct of experiment. The animals were weighed weekly during the first 13 wk and every 4 wk thereafter. Food intake was recorded only in wk 1, 2, 5, 9 and 12. Blood samples for haematological investigations were taken after 1 yr from ten animals in each group. The haemoglobin level, packed cell volume (PCV) numbers of erythrocytes and total and differential leucocytes were recorded. The mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH) and mean corpuscular haemoglobin concentration (MCHC) were calculated. Throughout the study any animals that became moribund were killed with carbon dioxide. All animals were inspected regularly for the presence of tumours. Extensive pathological examination was carried out on animals dying during the experiment and on the survivors, which were killed at month 27. The time at which each tumour was detected (during life or at autopsy) was noted. Organs were fixed in buffered 4% formalin or Bouin's solution, and the following organs and tissues were studied: brain, heart, lungs, liver, kidneys, spleen, pituitary gland, thymus (if any), thyroid, pancreas, adrenals, testes, ovaries, prostate, uterus, lymph nodes, salivary glands, gastro-intestinal tract (at six levels), urinary bladder, spinal cord, peripheral nerve, skeletal muscles, skin, mammary gland, tumours (if any) and any other tissue with a grossly observed abnormality.

Statistical methods. Statistical analysis was carried out on all measurements using Student's t test.

RESULTS

Growth, food intake and mortality

The means of the body weights recorded for each group at wk 6, 12, 20, 40, 60, 80, 100 and 120 are indicated in Table 2. The animals that received 416 ppm sodium arsenate alone or in combination with DENA and those that received 1850 ppm lead arsenate showed a lower body weight during the experiment.

The food intake was estimated at intervals only during the first 12 wk of the experiment (Table 2). The animals given 1850 ppm lead arsenate and both groups given 416 ppm sodium arsenate showed a significant decrease in food intake. The rats given DENA only increased their food intake compared with the controls.

The mortality (Table 3) was comparable in all groups except that given 1850 ppm lead arsenate. This group showed a marked increase in mortality after wk 26.

Table 2. *Mean body-weight gains and food intakes of rats fed lead arsenate or sodium arsenate alone or in combination with daily doses of DENA for up to 120 wk*

Group no.‡	Initial mean body weight§ (g)	Gain in weight‖ at wk								Food intake (g/day) at wk				
		6	12	20	40	60	80	100	120	1	2	5	9	12
						Males								
1a	99	165	233	271	308	336	341	317	280	14·4	16·3	17·4	18·9	16·9
1b	99	164	234	268	308	337	334	321	304	14·3	16·8	18·1*	19·8*	18·1**
2	76	140**	204**	236**	269**	283**	297**	238**	—	11·3**	12·4**	14·2**	16·4**	15·6*
3a	35	189	258	297	355	397	391	383	—	6·3	11·1	16·6	17·1	17·1
3b	99	170	241	271	321	342	340	307	293	14·4	16·8	18·3	20·3	18·6
4a	79	149**	212**	240**	282**	309**	316*	308	281	11·0**	12·8**	15·2**	17·6**	15·4**
4b	80	143	205†	230††	275††	304	308†	307	284	10·7††	12·6††	15·4††	17·9††	16·5•
						Females								
1a	82	81	114	132	147	170	187	190	176	11·7	12·2	11·9	13·2	11·6
1b	84	83	117	130	151	171	193	191	176	11·8	12·6*	12·8**	14·0**	12·7**
2	63	73**	103**	118**	136	148*	159*	145*	—	9·5**	9·6**	9·5**	10·6**	10·0**
3a	33	116	150	167	188	207	208	212	—	5·6	9·5	11·8	12·2	12·2
3b	84	82	117	133	156	180	194	182	164	11·4†	12·2	12·3†	13·7	12·5
4a	70	80	109	125*	142	157*	171*	169**	156	9·1**	9·7**	10·2**	11·9**	10·5**
4b	68	79	110†	123	144	159†	169††	165††	149†	8·8††	9·8††	10·4††	12·8††	11·0††

‡For treatments, see Table 1.
§Mean body weight at start of treatment.
‖Gain over initial body weight.
Values marked with superscripts differ significantly (Student's t test) from the corresponding value for group 1a (*$P < 0·05$; **$P < 0·01$), group 1b (†$P < 0·05$; ††$P < 0·01$) or group 4a (•$P < 0·05$).

Table 3. *Cumulative mortality of rats fed lead arsenate or sodium arsenate alone or in combination with DENA for up to 120 wk*

Group no.*	Initial group size	Cumulative mortality at wk									
		13	26	39	52	65	78	91	104	117	120
					Males						
1a	50	2	2	2	4	6	11	19	30	40	42
1b	50	1	3	4	5	6	13	18	29	41	44
2	29	0	0	2	3	8	12	18	27	29	29
3a	40	0	1	2	3	3	4	7	15	24	33
3b	40	1	2	2	2	5	6	13	28	36	37
4a	40	1	2	2	2	2	3	9	20	32	35
4b	40	2	2	2	2	3	4	12	20	26	30
					Females						
1a	60	1	1	1	1	1	2	6	9	22	25
1b	60	1	1	1	2	3	4	8	17	27	32
2	19	0	0	5	8	11	12	15	18	19	19
3a	40	0	1	1	1	1	1	4	9	21	28
3b	40	0	0	0	0	0	0	4	14	32	34
4a	40	1	1	1	2	2	3	8	22	30	30
4b	40	0	0	0	0	0	0	1	12	24	30

*Oesophageal intubation, 5 times/wk.

Table 4. *Haematological studies in rats fed lead arsenate or sodium arsenate alone or in combination with DENA for 52 wk*

Group no.‡	Hb (mmole/litre)	PCV (%)	RBC ($10^6/mm^3$)	MCV (fl)	MCH (amole/litre)	MCHC (mmole/litre)	Leucocytes: Total ($10^3/mm^3$)	Differential (%): B	E	N	L	M	A
Males													
1a	9·0	43·8	7·4	59·1	1289	21·9	9·0	0·5	2·4	19·8	73·6	3·7	3·0
1b	9·2	43·6	7·6	57·7	1223	21·2*	8·8	0·6	2·6	16·1	77·3	3·4	3·4
2	8·6**	41·4*	7·0	59·0	1226	20·8**	8·9	0·2	1·3*	24·0	72·0	2·5	2·4
3a	9·4	45·6	8·0	57·5	1187	20·7	9·1	0·1	1·4	14·4	82·7	1·4	1·8
3b	9·0	42·4	7·4	57·2	1206	21·1	9·8	0·4	1·4†	24·4†	71·0	2·8	3·2
4a	9·4	44·7	7·5	59·7	1259	21·1*	9·3	0·6	1·2**	19·0	76·6	2·6	3·6
4b	9·4	44·5	7·6	58·6	1243	21·2	9·2	0·7	1·7	14·0	81·2†	2·4†	3·7
Females													
1a	9·0	42·4	7·0	61·3	1298	21·1	7·1	0·4	1·3	14·4	81·0	2·9	3·8
1b	9·1	42·6	7·3	58·7	1254	21·4	6·3	0·1	1·2	19·3*	76·4*	3·0	3·2
2	8·7	42·0	6·8	62·1	1290	20·8	8·3	0·2	1·5	19·8	75·6	2·9	4·0
3a	9·3	44·5	7·3	60·7	1270	20·9	6·8	0·5	1·5	11·7	84·9	1·4	2·6
3b	9·0	42·5	7·4	57·3	1207	21·1†	6·9	0·4	1·4	14·2†	81·4	2·6	3·0
4a	9·3*	43·9	7·6*	58·2	1229	21·1	6·8	0·3	1·6	17·6	77·9	2·6	4·0
4b	9·1	43·5	7·0··	62·0	1302	21·0††	7·3	0·4	1·5	14·6	81·2	2·3	3·4

Hb = Haemoglobin PCV = Packed cell volume RBC = Red blood cells MCV = Mean cell volume MCH = Mean Hb/cell
MCHC = Mean corpuscular Hb concn B = Basophils E = Eosinophils N = Neutrophils L = Lymphocytes
M = Monocytes A = Atypical cells

‡For treatments, see Table 1.

Values are means for groups of ten animals and those marked with superscripts differ significantly (Student's t test) from the corresponding value for group 1a (*$P < 0·05$; **$P < 0·01$), group 1b (†$P < 0·05$; ††$P < 0·01$) or group 4a (··$P < 0·01$).

Haematology

Haematological studies were carried out at month 12 on ten rats from each group (Table 4). In the group given 1850 ppm lead arsenate the values for haemoglobin, PCV and the number of erythrocytes were generally lower than in the other groups, the values for haemoglobin and PCV in the males differing significantly from those of the controls. Other differences in the various parameters (Table 4), though significant, were not consistent and were therefore considered to be irrelevant.

Histopathology

All animals were studied histopathologically. The group of animals receiving 1850 ppm lead arsenate showed clearly the influence of a high dose of lead arsenate on the liver and kidneys. Macroscopically the changes consisted of a distinctly enlarged bile duct with dilatation and abscesses. Frequently bile concretions were found in the lumen of the bile duct and were assumed to be the cause of the dilatation and inflammation. The inflammation sometimes affected one of the regional blood vessels, causing intra-abdominal or intra-intestinal haemorrhages. This lesion was the cause of the high mortality in this group. Besides the extensive inflammation and dilatation of the bile duct, there was marked bile-duct proliferation in the liver, accompanied by pericholangitis and cholangiofibrosis.

Table 5. *Tumour incidence in rats fed lead arsenate or sodium arsenate alone or in combination with DENA for up to 120 wk*

Organ and lesion		No. of animals affected* in group†													
		1a		1b		2		3a		3b		4a		4b	
	Sex...	M	F	M	F	M	F	M	F	M	F	M	F	M	F
	Total no. of rats‡...	39	59	40	58	17	11	38	40	34	40	39	37	37	39
Heart															
Endotheliosarcoma										1					
Lung															
Adenomatosis								1		1					
Thorax															
Sarcoma			1												
Liver															
Hyperplastic area					2	1									
Hyperplastic nodules			1		1					1					
Small basophilic areas			2	2				1							
Bile duct															
Adenocarcinoma						1									
Kidney															
Cortical adenoma						1									
Hamartoma					1			1					1		
Nephroblastoma		(1)													
Spleen															
Lymphosarcoma				1											
Pituitary															
Chromophobic adenoma		1	13	3	14			2	8	1	7	3	7	2	4 (1)
Basophilic adenoma														1	
Lymphatic system															
Lymphangioma						1									
Lymphosarcoma				1	1										
Lymphatic leukaemia		(1)		1											
Lymphoblastosarcoma		1													
Myeloid leukaemia												1			

Table 5 (*cont.*)

Organ and lesion		No. of animals affected* in group†													
		1a		1b		2		3a		3b		4a		4b	
	Sex...	M	F	M	F	M	F	M	F	M	F	M	F	M	F
	Total no. of rats‡...	39	59	40	58	17	11	38	40	34	40	39	37	37	39
Thymus															
Thymoma											1				
Abdomen															
Sarcoma			1 (1)	(1)				(1)						1	
Adenocarcinoma (pancreas?)					(1)										
Thyroid															
Papillary carcinoma				2										1	
Medullary carcinoma				1	1								1		
Adrenal															
Cortical adenoma					1			2		1					1
Phaeochromocytoma		3	1	6	2	2		6	2	6	1	3	1	4	3
Malignant phaeochromocytoma								1							
Ovary															
Dysgerminoma									1						
Granulosa cell tumour			1		1						1				
Mammary gland															
Adenoma		(1)	1		5				1		1				1
Fibroadenoma			2		4						3		1		
Adenocarcinoma			1		2			1							
Tumour (gross examination)									2						
Uterus															
Anaplastic carcinoma													1		1
Adenocarcinoma					1				1						1
Carcinosarcoma			1												
Squamous cell carcinoma									[illegible]						
Testis															
Interstitial cell tumour		2						2		1		2			
Bladder															
Polypus											1				
Transitional cell carcinoma (Grade I)											1				2
Circulatory system															
Haemangiosarcoma										1					
Intestine															
Lipoma of caecal muscle			1						1						1
Pancreas															
Multiple adenomas or hyperplasia						1									
Adenocarcinoma											1				
Anaplastic carcinoma			1												
Insuloma								1	1						
Skin															
Adenoid-type basal cell epithelioma										1					
Fibroma		1												1	
Polymorphic sarcoma											1				
Sweat-gland adenosarcoma		1													
Peritoneum															
Carcinomatous lymphangitis															1

*Nos in parentheses refer to tumours found in animals that died before the age of 18 months.
†For treatments, see Table 1.
‡All rats that were killed and those that died and were found in a state fit for autopsy.

Intranuclear eosinophilic inclusions were seen in the kidneys, as previously described (Van Esch & Kroes, 1969), but the inclusions were present only in animals that died during the first 12 months of the test. No clear tubular degeneration was detected. In the other groups, there were no changes that could be ascribed to the administered compounds. The histological changes commonly found in rats were distributed equally among the groups. Although all abnormalities were recorded, only the tumours and hyperplastic lesions found in the animals that were killed and in those that died and were found in a state fit for autopsy have been summarized in Table 5. No specific tumours were clearly associated with any one of the groups. Only the adenoma of the renal cortex and the bile-duct carcinoma in the group given 1850 ppm lead arsenate could have been induced by the test compound. There were no major differences between the groups in the incidences of benign and malignant tumours (Table 6). Moreover, there appeared to be no differences between the groups in the times at which the tumours were observed, but this aspect may be questionable, since some tumours were detected only when the rats died or were killed for some reason other than the tumour.

Table 6. *Total incidence and time of observation of tumours in rats fed lead arsenate or sodium arsenate alone or in combination with DENA for up to 120 wk*

			Occurrence of tumours*					
			Malignant		Benign		Time of detection§ (day)	
Group no.†	Sex	No. of rats examined‡	Total no.	Incidence (%)	Total no.	Incidence (%)	Mean	Range
1a	M	39	4 (2)	10	8 (1)	21	698	319–889
	F	59	7 (1)	12	18	31	755	531–824
1b	M	40	7 (1)	17	9	22	771	584–913
	F	58	7 (1)	12	27	47	797	429–912
2	M	17	1	6	5	29	722	674–734
	F	11	0	0	0	0	—	—
3a	M	38	3 (1)	8	14	37	—‖	—‖
	F	40	3	7	15	37	—‖	—‖
3b	M	34	2	6	10	29	760	601–879
	F	40	4	10	14	35	783	689–856
4a	M	39	1	3	8	21	771	663–857
	F	37	2	5	10	27	744	579–863
4b	M	37	2	5	8	22	730	505–855
	F	39	5	13	11 (1)	28	783	548–889

*Totals given include tumours occurring before the age of 18 months, the actual numbers of which are given in parentheses.
†For treatments, see Table 1.
‡All rats that were killed and those that died and were found in a state fit for autopsy.
§Duration of experiment (in days) at the time the tumour was observed, whether the observation was made during life or at autopsy.
‖Due to a technical failure, the data for this group were lost.

DISCUSSION

It is clear that rats are unable to withstand a dose of 1850 ppm lead arsenate for a period of 2 yr. Histological changes attributable to the test compound were observed only in the group treated in this way. The observation that the intranuclear bodies in the kidney tubules disappeared as the experiment progressed is an interesting point, which has not

been noted before, except in so far as Choie & Richter (1972) mentioned that the inclusions were to be considered as an acute manifestation of lead intoxication. The alterations in the bile duct seem to be typical for lead arsenate.

The biosynthesis of haem is known to be influenced by lead (de Bruin & Hoolboom, 1967). If lead is administered in high doses, the haemoglobin content in the blood is decreased. The haematological results in this long-term study confirm this observation.

The cortical tumour found in the kidney of one rat in the group given the high dose of lead arsenate could possibly have been an indication of a weakly carcinogenic action of the lead in lead arsenate, for this observation is in accordance with previous findings by Boyland *et al.* (1962), Shakerin & Paloucek (1965), Van Esch & Kroes (1969) and Van Esch *et al.* (1962), who succeeded in demonstrating the carcinogenic action of lead acetate, which induced tumours in the renal cortex. The bile-duct carcinoma, too, may have been directly associated with the lead arsenate. However, the tumour incidence was far too low for any definite conclusion to be drawn with regard to the carcinogenicity of lead arsenate. There was no indication in this study that sodium arsenate acts as a carcinogen, and it appeared that DENA, in the dose used, was not able to induce tumours or to potentiate the action of lead arsenate or sodium arsenate. This may be an indication that there is a no-effect level for this carcinogen. The Wistar rats used in this experiment have a high spontaneous-tumour incidence. This could, on the one hand, diminish the value of the study, by increasing the possibility that a low incidence of induced tumours might pass unnoticed but, on the other hand, it suggests that the rat stock used may be particularly sensitive to the induction of tumours and/or their subsequent development. It may be concluded that this study produced no evidence of the carcinogenicity of sodium arsenate, while the indications were very uncertain for lead arsenate.

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REFERENCES

Boyland, E., Dukes, C. E., Grover, P. L. & Mitchley, B. C. V. (1962). The induction of renal tumours by feeding lead acetate to rats. *Br. J. Cancer* **16**, 283.

Choie, D. D. & Richter, G. W. (1972). Lead poisoning: Rapid formation of intranuclear inclusions. *Science, N.Y.* **177**, 1194.

de Bruin, A. & Hoolboom, H. (1967). Early sign of lead exposure. A comparative study of laboratory tests. *Br. J. ind. Med.* **24**, 203.

Frost, D. V. (1967). Arsenicals in biology—retrospect and prospect. *Fedn Proc. Fedn Am. Socs exp. Biol.* **26**, 194.

International Agency for Research on Cancer (1973). IARC Monographs on the Evaluation of the Carcinogenic Risk of Chemicals to Man. Some Inorganic and Organometallic Compounds. Vol. 2, p. 48. IARC, Lyon, 1973.

Lee, Anna M. & Fraumeni, J. F., Jr. (1969). Arsenic and respiratory cancer in man: An occupational study. *J. natn. Cancer Inst.* **42**, 1045.

Miller, J. A. & Miller, Elizabeth C. (1965). Natural and synthetic chemical carcinogens in the ethiology of cancer. (Compounds containing selenium, arsenic of various metals). *Cancer Res.* **25**, 1292.

Neubauer, O. (1947). Arsenical cancer: A review. *Br. J. Cancer* **1**, 192.

Shakerin, M. & Paloucek, J. (1965). Intranuclear inclusions and renal tumours in rat fed lead subacetate. *Lab. Invest.* **14**, 592.

Tseng, W. P., Chu, H. M., How, W. S., Fong, J. M., Lin, C. S. & Yeh, S. (1968). Prevalence of skin cancer in an endemic area of chronic arsenicism in Taiwan. *J. natn. Cancer Inst.* **40**, 453.

Vallee, B. L., Ulmer, D. D. & Wacker, W. E. C. (1960). Arsenic toxicology and biochemistry. *Archs ind. Hlth* **21**, 132.

Van Esch, G. J. & Kroes, R. (1969). The induction of renal tumours by feeding basic lead acetate to mice and hamsters. *Br. J. Cancer* **23**, 765.

Van Esch, G. J., Van Genderen, H. & Vink, H. H. (1962). The induction of renal tumours by feeding of basic lead acetate to rats. *Br. J. Cancer* **16**, 289.

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CARCINOGENIC EFFECTS OF LOW DIETARY LEVELS OF AFLATOXIN B_1 IN RATS

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Abstract—Aflatoxin B_1 was added to a semi-synthetic diet at levels of 1, 5, 15, 50 and 100 μg/kg (ppb). These diets were fed to male Fischer rats, beginning when their body weights were 80 g and continuing for up to 105 wk. A control group received the same diet without aflatoxin. Hepatocellular carcinomas were induced in a high incidence by levels of 50 and 100 ppb, and in a lower incidence by the other dietary levels. Tumours were induced in two of 22 animals fed 1 ppb, and pathological lesions were present in the livers of seven of the remaining survivors. These results suggest that, under these experimental conditions, the sensitivity of the Fischer rat to aflatoxin carcinogenesis is approximately the same as that previously reported for the rainbow trout.

INTRODUCTION

During the course of an earlier investigation of the dose-response relationships of aflatoxin B_1 carcinogenesis in the rat, we found that liver cancer was induced in every animal surviving longer than 50 wk when they were fed a highly purified diet containing pure aflatoxin B_1 added at a level of 15 ppb (Wogan & Newberne, 1967). These observations were made in rats of the Fischer strain and both males and females were affected similarly, although the tumours appeared after a longer latent period in females than in males. These results were unanticipated in the light of other evidence available to us at the time, and it seemed important to investigate further the effects of low dietary levels of aflatoxin B_1 in this rat strain. The present experiment was planned to verify the responsiveness under these experimental conditions and to explore further the dose-response relationship that might be expected at even lower dietary levels. For these purposes, the experimental design included dietary levels which ranged from 1 ppb to 100 ppb. In other respects, we attempted to reproduce the earlier experimental conditions as precisely as possible, including the use of the same diet, of the same aflatoxin preparation and of animals of the same strain.

EXPERIMENTAL

Material. Aflatoxin B_1 used in these experiments was a highly purified crystalline preparation, isolated and purified by previously described methods (Wogan & Newberne, 1967).

Animals. Animals were male Fischer rats obtained from A. R. Schmidt Co., Madison, Wisconsin. They were obtained as weanlings and were fed control diet until they weighed approximately 80 g, at which time the experimental treatment was started. Throughout the experiment, they were housed in individual cages and were provided daily with fresh

diet and water *ad lib*. Each rat was weighed weekly and was observed daily for the detection of any clinical deterioration.

Preparation of diets. The diet used was an agar-gel formulation, the composition of which has been previously reported (Wogan & Newberne, 1967). In the preparation of diet containing aflatoxin B_1 at levels of 15 ppb and higher, appropriate amounts of the crystalline compound were dissolved in reagent-grade acetone and thoroughly mixed with casein. A thin slurry of casein in an excess of the acetone solution was prepared in order to ensure uniform distribution of aflatoxin B_1 on the casein particles. After thorough mixing, the solvent was completely evaporated at room temperature and in darkness in order to minimize aflatoxin decomposition, which is known to be enhanced by light and heat. The contaminated casein thus prepared was then diluted with aflatoxin-free casein to the appropriate concentration for diet formulation. Diets containing lower aflatoxin levels were similarly prepared except that the acetone solution was added to the entire batch of dry diet ingredients (minus vitamins) in order to ensure uniform distribution. Final concentrations of aflatoxin B_1 in diets prepared in this fashion were verified by assay of the initial preparations, using a slightly modified version of the physico-chemical procedure described by Eppley (1966). Control and contaminated diets were prepared weekly and were stored at 5°C in tightly sealed plastics containers. In order to minimize the possibility of experimental error in the feeding of various dietary preparations, an appropriate amount of aflatoxin B_1 for each weekly batch of each dietary level was distributed into colour-coded vials, which were then used in diet formulation. Vials were prepared for the entire experiment at the beginning and were stored in the dark at freezer temperature until used.

Conduct of experiment. The levels of aflatoxin added to the diet were 1, 5, 15, 50 and 100 ppb (μg/kg). Feeding of the experimental diets continued until clinical deterioration of animals was observed, at which time all of the survivors in that treatment group were killed for examination. Detailed autopsies were performed on all animals that died or were killed. Histological examinations were made on paraffin sections of tissues fixed in buffered formalin and stained with haematoxylin and eosin.

RESULTS

The principal findings of the experiment are summarized in Table 1. In addition to the incidence of histologically confirmed liver-cell carcinoma, we have also included the occurrence of foci of hyperplasia and transitional cells which, on the basis of extensive experience with this experimental model, we regard to be early and intermediate stages, respectively, in the development of aflatoxin-induced carcinomas. Several features of the dose-response relationship over this range of dietary aflatoxin levels are evident in the data summarized in Table 1. A very high incidence of hepatocellular carcinoma occurred at the two highest aflatoxin levels. In addition, many of the surviving animals also had preneoplastic liver lesions. It is of particular interest that animals on the highest level (100 ppb) developed tumours substantially earlier than those fed 50 ppb.

The occurrence of fully-developed carcinoma at dietary levels of 15 ppb and lower was less well correlated with aflatoxin dose level. However, tumours were induced at each of the dietary levels studied, and included two confirmed carcinomas in a group of 22 animals fed the toxin at a level of 1 ppb. It is also noteworthy that in all three of the lower dietary levels, the livers of significant numbers of animals showed histological lesions, which we

Table 1. *Incidence of liver carcinoma and other liver pathology in rats as a function of dietary levels of aflatoxin B_1*

Aflatoxin level (ppb)	Time of appearance of earliest tumour (wk)	Duration of experiment (wk)	No. of animals at risk*	No. of animals with liver pathology		
				Hyperplasia	Transitional cells	Carcinoma
0		74–109	18†	1	0	0
1	104	78–105	22	6	1	2
5	93	65–93	22	4	1	1
15	96	69–96	21	13	0	4
50	82	71–97	25	8	7	20‡
100	54	54–88	28	8	4	28§

*Animals surviving longer than 50 wk.
†Animals surviving for maximum period.
‡Two rats with metastatic lesions in the lung.
§Four rats with metastatic lesions in the lung.

interpret to be preneoplastic. It can also be seen that lesions tended to develop more slowly in animals fed the lower dietary levels.

Rats fed the control diet showed no liver pathology, and the only tumours of tissues other than the liver found in either the control or aflatoxin-treated groups were interstitial-cell tumours of the testes, tumours which in this strain occur in a high incidence spontaneously and unrelated to aflatoxin treatment.

DISCUSSION

Several features of the results of this experiment are important to the matter of the carcinogenic potency of aflatoxin B_1 in rats. First, the results of this experiment in general confirm and extend our earlier findings. It is particularly noteworthy that carcinomas were induced at a dietary level of 15 ppb as in our earlier study. However, the incidence in the present study is smaller than that observed previously. The high incidence of carcinomas induced by the two higher dietary levels was anticipated on the basis of our own earlier results and of those from other laboratories.

The present results also indicate that aflatoxin B_1 has demonstrable carcinogenic activity for rat liver at dietary levels as low as 1 ppb. No other comparable studies have been carried out in rats at these low dietary levels. However, in terms of responsiveness, the results suggest that in this experimental model the sensitivity of the rat approaches that of the rainbow trout.

Several lines of evidence suggest that the experimental system employed here (male Fischer rats and a purified diet) may represent a much more sensitive system in the assay of aflatoxin carcinogenesis in rats than those employed by most other laboratories. There is a suggestion that both the rat strain and the purified diet may contribute to the increase in sensitivity.

Comparison of potency data from various laboratories suggests that Fischer rats may be more susceptible to both the acute and chronic effects of aflatoxins. No systematic comparisons have been made, but the possible importance of this factor is further emphasized by the results of two experiments conducted in our own laboratories in which a strain difference was apparent. In the present study, a dietary level of 100 ppb aflatoxin B_1 induced hepatocellular carcinomas in all of 28 animals. In a separate experiment in which aflatoxin

B_1 was fed in the same diet and at the same level, but to Sprague–Dawley rats, the incidence of tumours was only 24 out of 50 animals surviving for approximately the same length of time (Newberne & Rogers, 1973). While this obviously does not constitute definitive proof, it suggests that a more systematic comparison among rat strains might be worthwhile.

With respect to the possible importance of diet composition, information is available to suggest a higher responsiveness in rats fed purified diets than in those fed diets composed of natural ingredients. This is illustrated by data from the literature summarized in Table 2, which presents a combined summary of liver carcinoma incidence and dietary aflatoxin levels from published experiments in which continuous feeding was carried out for 1 yr or longer. All of these studies involved the incorporation into rat diets of peanut meals contaminated with aflatoxins under natural circumstances. Aflatoxin levels were determined by chemical assay, and were adjusted experimentally by dilution with uncontaminated meals. Although there was some minor variation in the aflatoxins present, aflatoxin B_1 was always the chief contaminant, and the results can be interpreted essentially as aflatoxin B_1 carcinogenesis.

Table 2. *Hepatocarcinogenicity to rats of diets containing aflatoxin-contaminated peanut meals*

Aflatoxin level (ppb)	Duration of exposure (wk)	Liver-tumour incidence	Reference
5	55	0/10	Newberne, Carlton & Wogan, 1964
10	104	0/10	Alfin-Slater, Aftergood, Hernandez, Stern & Melnick, 1969
70	58	1/7	Newberne, Harrington & Wogan, 1966
80	90	0/10	Alfin-Slater *et al.* 1969
100	50–80	17/34	Butler & Barnes, 1968
200	51–66	25/35	Newberne & Williams, 1969
400	51–87	15/27	Newberne *et al.* 1966
500	50–80	25/25	Butler & Barnes, 1968

Although the precise character of the dose-response curve cannot be inferred from these data, they are relatively consistent, considering the wide variations in experimental conditions. It is clearly evident that dietary aflatoxin levels of 100 ppb and higher induced liver carcinoma in an incidence greater than 50% when feeding was continued up to 80 wk. In the present context, the data obtained by feeding dietary levels below 100 ppb are of particular interest. It will be noted that induction of liver tumours occurred in only one experiment, with a dietary level of 70 ppb, and then in an incidence of only one in seven animals. Tumours were not induced by levels of 80 or 10 ppb in one experiment or in another by 5 ppb. Although the numbers of animals involved in these studies were not large and the information base is limited, the data strongly suggest that some feature of the natural diet results in a lower tumour-inducing potency than that observed in studies involving purified diets. A number of factors may contribute to this phenomenon including, for example, the possible presence of protective factors and/or poor absorption of bound aflatoxins. On the other hand, the possibility of sensitizing features in purified diets cannot be excluded, although none is readily apparent. No evidence is available on which to suggest the likely operative mechanism.

It is important to evaluate these and related data in the context of their relevance to the assessment of public health hazards associated with aflatoxin exposure of human populations. From the present experiment, as well as many previous publications, it is clear that aflatoxins are very potent liver carcinogens for most animal species, and exposure of man to any level must be regarded as representing some carcinogenic risk. However, the limited data on intake and liver-cancer incidence available from studies in human populations suggest that direct extrapolation of potency information from animals to man is unwarranted. In populations consuming aflatoxins at dietary levels of the same order of magnitude as those shown to be effective carcinogenic levels in rats, the incidence of liver carcinoma is far lower than would be expected on the basis of the animal data.

Nonetheless, every effort should be expended to minimize exposure whenever possible. Total elimination of aflatoxins from all dietary components may prove to be difficult or impossible to achieve owing to the circumstances under which they are introduced into food commodities. As analytical methodology permits detection of lower levels of contamination, technological processes for the complete prevention of contamination may become ineffective or so expensive as to impair or destroy the utility of important foods. In this circumstance, continued use of foods will necessarily depend on risk-benefit analyses, taking into account the scientific consideration of the other elements relevant to the evaluation.

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REFERENCES

Alfin-Slater, Roslyn B., Aftergood, Lilla, Hernandez, H. J., Stern, Elizabeth & Melnick, D. (1969). Studies of long term administration of aflatoxin to rats as a natural food contaminant. *J. Am. Oil Chem. Soc.* **46,** 493.

Butler, W. H. & Barnes, J. M. (1968). Carcinogenic action of groundnut meal containing aflatoxin in rats. *Fd Cosmet. Toxicol.* **6,** 135.

Eppley, R. M. (1966). A versatile procedure for assay and preparatory separation of aflatoxins from peanut products. *J. Ass. off. analyt. Chem.* **49,** 1218.

Newberne, P. M., Carlton, W. W. & Wogan, G. N. (1964). Hepatomas in rats and hepatorenal injury in ducklings fed peanut meal or Aspergillus flavus extract. *Path. vet.* **1,** 105.

Newberne, P. M., Harrington, D. H. & Wogan, G. N. (1966). Effects of cirrhosis and other liver insults on induction of liver tumors by aflatoxin in rats. *Lab. Invest.* **15,** 962.

Newberne, P. M. & Rogers, A. R. (1973). Rat colon carcinomas associated with aflatoxin and marginal vitamin A. *J. natn. Cancer Inst.* **50,** 439.

Newberne, P. M. & Williams, Gail (1969). Inhibition of aflatoxin carcinogenesis by diethylstilbestrol in male rats. *Archs envir. Hlth* **19,** 489.

Wogan, G. N. & Newberne, P. M. (1967). Dose-response characteristics of aflatoxin B_1 carcinogenesis in the rat. *Cancer Res.* **27,** 2370.

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THE AETIOLOGY OF CAECAL ENLARGEMENT IN THE RAT

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Abstract—The effect of hydroxypropyl starches (degree of substitution (DS) 0·025–0·106), lactose, raw potato starch, polyethylene glycol 1000 or magnesium sulphate on caecal size and some constituents of the caecal contents was studied in male rats of ages varying from 4 wk to 3 months in experiments lasting from 10 days to 3 months. All the test compounds induced caecal enlargement under the experimental conditions. Caeca enlarged by a hydroxypropyl starch (DS 0·047), lactose or raw potato starch, returned to normal sizes within 4 wk after the animals reverted to a control diet. The analytical data did not show a consistent relationship between caecal size and the percentages of dry matter, sodium, potassium, chloride or volatile fatty acids in the caecal contents. The osmotic values of the caecal contents of control and experimental groups were of the same order of magnitude. It is postulated that the size of the rat caecum is controlled by the osmotic value of the caecal contents, irrespective of the nature or origin of the compounds contributing to this value, and the conclusion is drawn that caecal enlargement is a process of physiological adaptation.

INTRODUCTION

Chemically modified starches are used in the food industry as thickeners, binders, stabilizers and film-forming agents. Several types are used, including starch acetates, starch phosphates, hydroxypropyl starches and cross-linked varieties of these products (Vogel, 1969; Wurzburg & Szymanski, 1970).

In the safety evaluation of the various starch derivatives, extensive toxicological studies have been carried out in rats, and the most striking effect seen in the studies has been a more or less marked enlargement of the caecum, sometimes accompanied by diarrhoea (de Groot, Til, Feron, Dreef-van der Meulen & Willems, 1974; Joint FAO/WHO Expert Committee on Food Additives, 1972). This effect was common to most of the products examined irrespective of the way the chemical modification was brought about, although it was apparently dependent on the degree of modification. The aetiology of the phenomenon has never been systematically studied. It has been suggested that the enlargement is due to an increase in the bulk of the caecal contents (Joint FAO/WHO Expert Committee on Food Additives, 1972).

Besides the chemically modified starches, a number of other compounds are known to have an effect on the caecal size. These include sorbitol (Morgan & Yudkin, 1957), lactose and several raw starches (Reussner, Andros & Thiessen, 1963), maltitol (Hosoya, 1972), a high-molecular-weight fraction of glucose syrup (Birch & Etheridge, 1973), polyethylene glycol (Loeschke, Uhlich & Halbach, 1973) and magnesium sulphate (Moinuddin & Lee, 1960).

To obtain a more detailed insight into the factors controlling caecal size, we examined what effect the dietary concentration and degree of modification of several hydroxypropyl starches had on the caecum and compared a number of biophysical and biochemical measurements of the caecal contents following administration of various caecum-enlarging compounds. Hydroxypropyl starch was chosen as a model substance for the group of chemically modified starches, as it had been found to have a more pronounced effect on caecal size than the other modified starches we had examined. The results reported here indicate that the size of the caecum is controlled by osmotic processes.

EXPERIMENTAL

Caecum-enlarging compounds. The two types of modified starch used were hydroxypropyl starch, obtained from potato starch by treatment with propylene oxide in an alkaline solution (Hjermstad, 1967) and with a degree of substitution (DS) varying from 0·025 to 0·106, and a hydroxypropyl distarch glycerol, obtained from potato starch by cross-linking with 0·1% epichlorohydrin and subsequent etherifying with propylene oxide and with a DS of 0·05. The products were prepared and pre-gelatinized by drum-drying by Scholten-Honig Research B. V., Foxhol, The Netherlands. A modified Zeisel-Morgan procedure was used to determine the DS (Van der Bij, 1967). The other compounds used to produce caecal enlargement were raw potato starch, lactose, anhydrous magnesium sulphate and polyethylene glycol 1000 (BDH Chemicals Ltd., Poole, Dorset; degree of polymerization 19–22).

Animals and diets. The studies were carried out with male rats from the Institute's Wistar-derived colony. In three experiments (A, B and C) the control diet was a semi-synthetic diet of the following percentage composition: Casein, 18; sucrose, 16·8; minerals (Jones & Foster, 1942), 4; complete B-vitamin mixture, 0·2; 50% choline preparation, 0·4; vitamin-ADEK preparation, 0·4; D,L-methionine, 0·2; soya-bean oil, 5; cellulose powder (Solka Floc), 5; unmodified potato or corn starch, pre-gelatinized by drum-drying, 50. In the fourth experiment (D) the control diet had the following percentage composition: Fish meal, 8; meat scraps, 4; soyabean-oil meal, 20; whole wheat, 13; bone meal, 1; trace minerals in salt, 0·4; complete B-vitamin mixture, 0·2; vitamin-ADEK preparation, 0·4; margarine, 3; unmodified potato or corn starch, pre-gelatinized by drum-drying, 50. In the experimental diets an appropriate part of the control starch was replaced with an equal amount of a compound inducing caecal enlargement. Diets and tap-water were provided *ad lib.*

Experimental design and conduct

Experiment A. Groups of ten male weanling rats were fed semi-purified diets in which 10, 20, 30 or 40% of the pre-gelatinized potato starch was replaced by a hydroxypropyl starch of DS 0·025, 0·047, 0·067 or 0·106. In this experiment the 5% of cellulose powder was omitted from the diet and the sucrose concentration was increased to 21·8%. One group was placed on the control diet. The degree of diarrhoea was estimated by a subjective grading system. After 10 days the animals were weighed and killed by decapitation, and the filled and empty caeca were weighed. The autopsies were performed between 9.00 and 11.00 hr.

Experiment B. Eighty-seven young, male rats (70–90 g body weight) were adapted to the semi-purified diet containing pre-gelatinized potato starch for 1 wk. They were then

divided into one control group of 15 animals and six experimental groups each of 12 animals. Three of the latter groups received diets in which 10, 30 or 50% of the control starch was replaced by the hydroxypropyl distarch glycerol preparation, and the other three groups received diets in which 5, 15 or 25% of the unmodified potato starch was replaced by lactose. The water consumption was monitored throughout the experiment and the degree of diarrhoea was estimated. After 2 wk the animals were weighed and killed by decapitation. The filled and empty caeca were weighed and the caecal contents were collected. The caecal contents from each dietary group were pooled and thoroughly mixed prior to further analysis. The autopsies were performed between 9.00 and 11.00 hr.

Experiment C. Thirty-six male rats (2·5 months old, 190–260 g body weight) were divided into three groups each of 12 animals. One group was given the control diet, containing 50% pre-gelatinized corn starch, and tap-water, the second group received the control diet and a 4% (w/v) solution of polyethylene glycol 1000 as drinking-water, and the third group received the control diet with 3% of the starch replaced by anhydrous magnesium sulphate, and tap-water. The water consumption was recorded in wk 3. After 3 wk the animals were killed by decapitation and dissected as described above.

Experiment D. The animals were given the practical-type diet, containing fish meal. The control diet contained 50% pre-gelatinized potato starch, while in the three test diets 40, 30 and 40% of the latter starch was replaced by raw potato starch, lactose and hydroxypropyl starch (DS 0·047), respectively. Eighty male weanling rats were divided and housed in groups of five. Each diet was given to four of these groups. After 3 months, ten animals from each dietary group were weighed, killed by decapitation and dissected as described above. The remaining animals were placed on the practical-type diet containing 50% pregelatinized corn starch for another month. The animals were then weighed, killed and dissected.

Analytical methods

The percentage dry matter of the caecal contents was determined by freeze-drying an aliquot of the material collected at autopsy. The freeze-dried material was used for the determination of sodium, potassium and chloride. Sodium and potassium were determined by flame photometry or atomic absorption photometry of solutions obtained by extraction of 100 mg sample with 10 ml hot 4 N-HCl, followed by dilution with an appropriate volume of demineralized water and filtration. Chloride was determined by coulometric titration with a Chlor-o-counter (Marius, Utrecht) of extracts prepared by extraction of 500 mg sample with 20 ml hot demineralized water, followed by deproteinization and de-fatting according to Carrez (1909), dilution to 50 ml with demineralized water and filtration.

The volatile fatty acid content was determined in supernatants obtained by treating aqueous suspensions of aliquots of the wet caecal contents with $FeCl_3$ and $Ca(OH)_2$ (Weijers, Van de Kamer, Dicke & IJsseling, 1961) followed by centrifugation. These supernatants were subjected to steam distillation and titration. The fatty acid composition of the neutralized distillate was analysed by gas-liquid chromatography (Van de Kamer, Gerritsma & Wansink, 1955).

Osmolalities were calculated from the melting curves of the supernatants obtained by centrifugation of homogenates of wet caecal contents and demineralized water (1 : 1, v/v), using the method of Smit, Ruyter & van Wijk (1960). The pH of these supernatants was determined by means of Lyphan strips L 665 or L 669.

RESULTS

Experiment A

The data presented in Table 1 clearly show that the relative caecal weights, both filled and empty, increased with increasing dietary concentration of the various hydroxypropyl starches. The same applied to the severity of diarrhoea. Although not strictly proportional, the increases in filled and empty caecal weight also showed a positive correlation with DS.

Experiment B

Besides caecal weights and the severity of diarrhoea, a number of biophysical and biochemical measurements were made on the caecal contents and the water consumption was determined. The results are presented in Table 2. The determination of sodium, potassium and chloride was included in the experiment because, under normal conditions, these are the major inorganic electrolytes of the intestinal tract. The volatile fatty acids were chosen as indicators of bacterial activity. In preliminary experiments it was found that the caecal lactic acid concentration was relatively low, usually < 1 μequiv/g wet weight. Therefore, this acid was not determined in this and the following experiments.

The data in Table 2 show a dose-related effect on caecal size and severity of diarrhoea. The sodium, potassium and chloride concentrations were decreased in the groups given hydroxypropyl distarch glycerol. In the lactose groups the potassium concentration was increased and at the highest dose the chloride concentration was decreased. The volatile fatty acid concentrations were slightly increased in all experimental groups. The water consumption was increased in the hydroxypropyl distarch glycerol groups, but only slightly increased in the lactose groups. These effects were dose-related to a limited extent. The osmotic activity was increased in all experimental groups.

One of the most important features of this experiment was the marked decrease in the sum of the sodium, potassium, chloride and volatile fatty acid concentrations in the groups given hydroxypropyl distarch glycerol.

Table 1. *Caecal enlargement in male, weanling rats fed for 10 days on semi-synthetic diets containing various amounts of hydroxypropyl starches of different DS (Experiment A)*

Diet		(%)	Occurrence of diarrhoea*	Mean relative caecal weight (g/100 g body weight)	
				Filled	Empty
Control starch			–	1·18	0·26
Hydroxypropyl starch	DS 0·025	30	+	2·26	0·40
	DS 0·047	10	–	1·29	0·31
		20	+	1·73	0·36
		30	+ +	2·16	0·48
		40	+ + +	2·82	0·68
	DS 0·067	20	+ +	2·45	0·40
		30	+ + +	3·01	0·47
	DS 0·106	20	+ +	2·76	0·40
		30	+ + +	3·63	0·62

DS = Degree of substitution

*Grading system: – absent; + slight; + + moderate; + + + severe.

Table 2. *Analysis of caeca of young male rats, fed semi-synthetic diets containing various concentrations of hydroxypropyl distarch glycerol (DS 0·05) or lactose for 2 wk (Experiment B)*

Parameter	Values for animals fed						
	Control diet	Hydroxypropyl distarch glycerol diet			Lactose diet		
Dietary concn (%)...		10	30	50	5	15	25
Water consumption (ml/animal/day)	15·1	18·4	21·5	23·5	17·4	13·9	18·8
Diarrhoea*							
Wk 1	–	–	+ +	+ + +	–	+	+ +
Wk 2	–	–	+ +	+ + +	–	–	–
Relative caecal weight (g/100 g body weight)							
Filled	1·05	1·29	2·67	4·39	1·16	1·79	2·52
Empty	0·27	0·27	0·46	0·65	0·28	0·35	0·53
Caecal contents							
Dry matter (%)	29·2	30·0	27·6	22·0	28·6	24·9	24·4
pH	7·2	6·6	5·8	5·8	6·6	7·5	6·6
Osmotic value (mosmol/kg caecal fluid)	310	370	350	320	320	360	370
Volatile fatty acids (μequiv/g wet weight)	118	135	156	157	148	150	158
Sodium (μequiv/g wet weight)	118	88	44	45	99	107	92
Potassium (μequiv/g wet weight)	25	22	15	11	34	44	43
Chloride (μequiv/g wet weight)	18	13	7	6	17	17	12
Sum (μequiv/g wet weight)	279	258	222	219	298	318	305

DS = Degree of substitution

*Grading system: – absent; + slight; + + moderate; + + + severe.

Experiment C

The main objective of this experiment was to examine what effect a non-digestible and non-absorbable product like polyethylene glycol and a poorly absorbable salt like magnesium sulphate would have on the various parameters determined in the previous study. The results, presented in Table 3, show that both products caused considerable caecal enlargement but that only polyethylene glycol induced severe diarrhoea. The sodium, potassium and volatile fatty acid concentrations were decreased in both experimental groups and the water consumption was slightly increased. The chloride concentration was decreased only in the polyethylene glycol group. The differences in volatile fatty acid composition are probably of minor importance, acetic acid being by far the major component in the groups.

Experiment D

This experiment was carried out to establish whether caecal enlargement, once induced, would be reversible after replacement of the caecum-enlarging compound by an unmodified, pre-gelatinized starch. A practical-type diet was used to allow a more meaningful comparison with the toxicity studies carried out on a number of chemically modified starches (de Groot *et al.* 1974).

The results obtained in animals fed the caecum-enlarging components for 3 months are summarized in Table 4. In all experimental groups the total fatty acid concentration was decreased and acetic acid was again the major component. The differences in potassium

Table 3. *Analysis of caeca of adult male rats fed polyethylene glycol (PEG) 1000 or magnesium sulphate for 20 days (Experiment C)*

Parameter	Values for animals given		
	Control diet	4% PEG 1000 (in drinking-water)	3% $MgSO_4$ (in diet)
Water consumption in wk 3 (ml/animal/day)	29·8	35·5	34·0
Diarrhoea*	–	+ + +	±
Relative caecal weight (g/100 g body weight)			
Filled	0·74	2·48	2·14
Empty	0·29	0·40	0·37
Caecal contents			
Dry matter (%)	25·8	23·8	15·8
pH	7·3	6·8	7·2
Osmotic value (mosmol/kg caecal fluid)	360	390	260
Volatile fatty acids (μequiv/g wet weight)			
Formic acid	3	2	1
Acetic acid	76	15	39
Propionic acid	23	2	10
Isobutyric acid	1	–	–
Butyric acid	14	1	1
Isovaleric acid	1	–	–
Valeric acid	2	1	1
Caproic acid	–	–	–
Total	120	21	52
Sodium (μequiv/g wet weight)	100	24	8
Potassium (μequiv/g wet weight)	21	10	11
Chloride (μequiv/g wet weight)	19	5	17
Sum (μequiv/g wet weight)	260	60	88

*Grading system: – absent; ± very slight; + + + severe.

Table 4. *Analysis of caeca of male rats fed for 3 months after weaning on practical-type diets containing different caecum-enlarging compounds (Experiment D)*

Parameter	Values for animals fed			
	Control diet	Raw potato starch (40%)	Lactose (30%)	Hydroxypropyl starch (40%)
Relative caecal weight (g/100 g body weight)				
Filled	1·03	3·22	2·66	2·34
Empty	0·26	0·57	0·55	0·40
Caecal contents				
Dry matter (%)	23·4	26·5	19·7	24·0
pH	6·6	5·8	6·6	5·6
Osmotic value (mosmol/kg caecal fluid)	440	450	410	450
Volatile fatty acids (μequiv/g wet weight)				
Formic acid	2	2	—	11
Acetic acid	132	102	100	82
Propionic acid	43	26	57	29
Isobutyric acid	1	—	—	—
Butyric acid	35	30	19	16
Isovaleric acid	1	—	—	1
Valeric acid	4	2	2	2
Caproic acid	—	3	—	—
Total	218	165	178	141
Sodium (μequiv/g wet weight)	72	98	85	40
Potassium (μequiv/g wet weight)	30	26	37	26
Chloride (μequiv/g wet weight)	11	10	12	6
Sum (μequiv/g wet weight)	331	299	312	213

concentration were probably not significant. The sodium and chloride concentrations were comparable in the groups given the control diet and diets containing raw potato starch and lactose, but were markedly decreased in the hydroxypropyl starch group. The sum of sodium, potassium, chloride and volatile fatty acid concentration was also decreased in this group.

The results obtained after a recovery period of 4 wk are presented in Table 5 and show that by this time there were almost no differences between the four groups as regards caecal size and the other parameters studied. This also applied to the volatile fatty acid composition of the various groups and therefore the results of these analyses are not shown.

DISCUSSION

The results of the present studies confirm again that caecal enlargement can be induced by a variety of compounds and that the phenomenon is dose-related.

The main objective of experiment A was to study the relationship between caecal size and the digestibility of hydroxypropyl starches. In a previous study the *in vitro* digestibility of such starches with pancreatin was found to decrease exponentially with increasing DS (Leegwater & Luten, 1971). It seemed reasonable, therefore, to assume that the same would apply to the *in vivo* digestibility. The *in vitro* digestibilities with pancreatin were 86, 78, 71 and 57% for the four products with DS 0·025, 0·047, 0·067 and 0·106, respectively, the digestibility of unmodified, pre-gelatinized potato starch being taken as 100%. The digestibilities predicted from a model for the structure of hydroxypropyl starches (Leegwater, 1972), namely 87, 78, 65 and 55%, respectively, were in fair agreement with these results.

Table 5. *Analysis of caeca of male rats fed for 3 months on practical-type diets containing different caecum-enlarging compounds and subsequently fed the control diet for 1 month (Experiment D)*

Parameter	Values for animals fed for 3 months on			
	Control diet	Raw potato starch (40%)	Lactose (30%)	Hydroxypropyl starch (40%)
Relative caecal weight (g/100 g body weight)				
Filled	0·98	1·02	0·96	0·99
Empty	0·24	0·29	0·25	0·23
Caecal contents				
Dry matter (%)	23·9	23·2	23·4	23·5
pH	6·6	6·6	6·6	6·6
Osmotic value (mosmol/kg caecal fluid)	520	520	530	510
Total volatile fatty acids (μequiv/g wet weight)	242	213	231	230
Sodium (μequiv/g wet weight)	79	79	80	80
Potassium (μequiv/g wet weight)	27	32	30	28
Chloride (μequiv/g wet weight)	11	11	11	12
Sum (μequiv/g wet weight)	359	335	352	350

As pointed out earlier, chemically modified starches may induce diarrhoea as well as caecal enlargement. The data in Table 1 show that the various hydroxypropyl starches had a clearly dose-related effect on both caecal size and the severity of diarrhoea. This seems to indicate that the two phenomena are interrelated. However, the data in Tables 2 and 3 show that although, under the conditions applied, hydroxypropyl distarch glycerol, lactose, polyethylene glycol and magnesium sulphate induced a comparable degree of caecal enlargement, at the end of the various test periods only the animals on hydroxypropyl distarch glycerol and polyethylene glycol still had severe diarrhoea. The animals on lactose had diarrhoea in wk 1 but not in wk 2 of the study, while most of the animals on magnesium sulphate were free from diarrhoea throughout the experiment, only a few rats giving soft droppings. From these observations it appears that the development of diarrhoea depended on the type of compound responsible for the caecal enlargement.

Of the caecum-enlarging compounds mentioned in the Introduction, raw potato starch and lactose are incompletely digested in the small intestine of the rat but are metabolized extensively by the gut bacteria. Since chemically modified starches and the high molecular weight fraction of glucose syrup most probably behave in the same way, it seems plausible to assume that caecal enlargement is due to an increase in bacterial activity. However, the fact that the enlargement can also be induced by a metabolically inert product like polyethylene glycol or an inorganic salt like magnesium sulphate indicates that the phenomenon is due rather to osmotic processes.

The following features of the present studies support the latter possibility. The experimental conditions varied with regard to the type of basic diet, the age of the animals, the duration of the experiment and the nature of the caecum-enlarging compounds. When one compares the data obtained in the various experiments, it appears that any changes in the concentration of the caecal components examined were not related to the degree of caecal enlargement. This is illustrated by the following examples.

The sodium concentration was lowered by the various hydroxypropyl starches, polyethylene glycol and magnesium sulphate but hardly affected by lactose or raw potato starch. The potassium concentration was decreased in the hydroxypropyl starch, polyethylene

glycol* and magnesium sulphate groups on the semi-synthetic diets, increased in the lactose groups on the semi-synthetic diet, but hardly affected by raw potato starch, lactose or hydroxypropyl starch incorporated in the practical-type diets. The chloride concentration was decreased in all hydroxypropyl starch groups, the polyethylene glycol group and the 25% lactose group on the semi-synthetic diet, but not distinctly changed in the other lactose groups or in the raw potato starch and magnesium sulphate groups. The concentration of volatile fatty acids was elevated in the groups given hydroxypropyl distarch glycerol or lactose in the semi-synthetic diets, but was lowered by raw potato starch, lactose and hydroxypropyl starch in the practical-type diet and also by polyethylene glycol and magnesium sulphate in the semi-synthetic diet. The pH of the caecal fluids varied from experiment to experiment, and the percentage of dry matter in the caecal contents varied from 16–30% without any correlation with caecal size.

The osmotic values, on the other hand, showed a more consistent pattern. In experiment B, the values for the test groups were up to 20% higher than the osmotic value of the control group. In experiment C, the control and polyethylene glycol groups had approximately the same osmotic value, but this value was about 30% lower for the magnesium sulphate group. In experiment D, the osmotic values for the control and test groups were approximately the same. So, with the exception of magnesium sulphate, the osmotic values of the test groups were of the same order of magnitude as those of the corresponding control groups or were somewhat increased. It should be noted that the osmotic values of the controls varied from 310 to 520 mosmol/kg caecal fluid in the various experiments. It has not been established whether this variation was due to the use of dilutions of the aqueous phase in the determination of the osmotic values (resulting in a further dissociation of the fatty acids present) or to the different diets or age of the animals. However, for the present discussion this fact is of secondary importance, the relevant point being that in each of the experiments the osmotic values of control and test groups were comparable.

The data in Tables 2–5 show that sodium, potassium, chloride and the volatile fatty acids accounted for the major part of the osmotic activity of the caecal fluid of the animals on the control, lactose or raw potato starch diets. By contrast, their contribution to the osmotic value of the caecal contents of the animals on the various hydroxypropyl starch diets and on the polyethylene glycol and magnesium sulphate diets was consistently less. However, it should be noted that hydroxypropyl starches yield a substantial quantity of metabolites notably hydroxypropyl maltoses (Leegwater & Speek, 1972), which are not absorbed (D.C. Leegwater, unpublished observation 1971). Polyethylene glycol is not absorbed either and an excessive amount of magnesium sulphate is only partially absorbed (a preliminary experiment established that about 70% of the magnesium consumed by the rats in experiment C was excreted in the faeces). Hence, it is obvious that a large proportion of the osmotic activity of the caecal contents of rats on diets containing the latter caecum-enlarging compounds was due to these non-absorbed or poorly absorbed compounds.

On the basis of the above considerations we present, therefore, as a working hypothesis: The size of the caecum is controlled by the osmotic value of the caecal contents, irrespective of the nature or origin of the compounds contributing to this value.

*It should be mentioned that Loeschke *et al.* (1973) in their studies with polyethylene glycol 4000 did not observe the decreased sodium and potassium concentration found in the present study with polyethylene glycol 1000.

Starting from this hypothesis the aetiology of caecal enlargement can be described as follows: Dietary components which are not completely digested and/or absorbed in the small intestine give rise to an increased amount of osmotically active material in the intestinal contents. This amount may increase further in the caecum if the non-absorbed fragments can be utilized by the gut bacteria as a substrate and if this results in the production of low molecular weight metabolites which are not, or are not readily, absorbed. The increase in the amount of osmotically active substances results in an increase in water retention (the animals tend to drink more) and the caecum distends to a size larger than normal.

As mentioned in the Introduction, it has been suggested that caecal enlargement is due to an increase in the bulk of the caecal contents. It should be noted that in our conception it is not the bulk as such which induces the enlargement, but rather its osmotic activity. This view is supported by the results of an investigation in which no distinct effect on the caecal size was found when diets containing up to 40% of indigestible polyethylene powder were given to young rats for a period of 4 wk (Spanjers, 1971).

In connexion with the safety evaluation of chemically modified starches (Joint FAO/WHO Expert Committee on Food Additives, 1972), the question arises whether caecal enlargements of the type discussed are of toxicological significance. It should be noted that in all the studies carried out so far, no histological evidence has been obtained indicating that the feeding of chemically modified starches affects caecal morphology (de Groot *et al.* 1974; Joint FAO/WHO Expert Committee on Food Additives, 1972). Furthermore, it should be recalled that in experiment D the caecum returned to a normal size within 4 wk when the caecum-enlarging component in the diet was replaced by unmodified, pregelatinized starch.

In view of these results we conclude that caecal enlargement is merely a physiological adaptation process which in itself is of no toxicological significance. However, side-effects, like severe diarrhoea, may have an untoward effect on the well-being of the animal.

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REFERENCES

Birch, G. G. & Etheridge, I. J. (1973). Chemical and physiological properties of glucose syrup components. *Stärke* **25,** 235.

Carrez, C. (1909). Défécation du lait pour le dosage du lactose par les liqueurs cupriques. *Ann. Chim. anal. Rev. Chim. anal.* **14,** 187.

de Groot, A. P., Til, H. P., Feron, V. J., Dreef-van der Meulen, Harriet C. & Willems, Marian I. (1974). Two-year feeding and multigeneration studies in rats with five chemically modified starches. *Fd Cosmet. Toxicol.* **12,** 651.

Hjermstad, E. T. (1967). Production and uses of hydroxyethylstarch. In *Starch: Chemistry and Technology*. Vol. II. Edited by R. L. Whistler and E. F. Paschall. p. 423. Academic Press, New York.

Hosoya, N. (1972). Effect of sugar alcohol on the intestine. IX International Congress of Nutrition, Mexico City.

Joint FAO/WHO Expert Committee on Food Additives—Fifteenth Report (1972). Evaluation of Food Additives. Some Enzymes, Modified Starches, and Certain other Substances: Toxicological Evaluations and Specifications and a Review of the Technological Efficacy of some Antioxidants. *Tech. Rep. Ser. Wld Hlth Org.* **488.**

Leegwater, D. C. (1972). A model for the structure of hydroxypropylstarches. *Stärke* **24,** 11.

Leegwater, D. C. & Luten, J. B. (1971). A study on the in vitro digestibility of hydroxypropylstarches by pancreatin. *Stärke* **23,** 430.

Leegwater, D. C. & Speek, A. J. (1972). Study on the faecal metabolites of hydroxypropylstarch in the rat. *Stärke* **24,** 373.

Loeschke, K., Uhlich, E. & Halbach, Regine (1973). Cecal enlargement combined with sodium transport stimulation in rats fed polyethylene glycol. *Proc. Soc. exp. Biol. Med.* **142**, 96.
Moinuddin, J. F. & Lee, H. W. T. (1960). Alimentary, blood and other changes due to feeding $MnSO_4$, $MgSO_4$ and Na_2SO_4. *Am. J. Physiol.* **199**, 77.
Morgan, T. B. & Yudkin, J. (1957). The vitamin-sparing action of sorbitol. *Nature, Lond.* **180**, 543.
Reussner, G., Andros, J. & Thiessen, R. (1963). Studies on the utilization of various starches and sugars in the rat. *J. Nutr.* **80**, 291.
Smit, W. M., Ruyter, J. H. & van Wijk, H. F. (1960). A new cryoscopic micro-method for the determination of molecular weights. *Analytica chim. Acta* **22**, 8.
Spanjers, M. Th. (1971). Onderzoek naar de waarde van natuurlijke en gezuiverde ruwvezelcomponenten in rantsoenen voor ratten. CIVO-TNO rapport No. R 3577.
Van de Kamer, J. H., Gerritsma, K. W. & Wansink, E. J. (1955). Gas liquid partition chromatography: the separation and micro-estimation of volatile fatty acids from formic acid to dodecanoic acid. *Biochem. J.* **61**, 174.
Van der Bij, J. R. (1967). Moderne Analysemethoden für Stärkederivate und Stärkehydrolysate. *Stärke* **19**, 256.
Vogel, W. F. (1969). Gemodificeerde zetmelen en hun toepassing in de voedingsmiddelenindustrie. *Conserva* **18**, 1.
Weijers, M. A., Van de Kamer, J. H., Dicke, W. K. & IJsseling, J. (1961). Diarrhoea caused by deficiency of sugar splitting enzymes. I. *Acta paediat. Stockh.* **50**, 55.
Wurzburg, O. B. & Szymanski, C. D. (1970). Modified starches for the food industry. *J. agric. Fd Chem.* **18**, 997.

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SHORT PAPER

SANGUINARINE IN THE BLOOD AND URINE OF CASES OF EPIDEMIC DROPSY

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Summary—During a recent outbreak of epidemic dropsy at Sirpur-Kagaznagar, Andhra Pradesh, samples of blood and urine were obtained from patients severely affected with symptoms of the disease and oil samples were collected from their households. Screening of these samples for the presence of sanguinarine indicated that all the household samples of oil, all but one of the 22 random urine samples and two of the 22 blood samples taken were positive. The two blood samples contained 4·7 and 28·3 μg sanguinarine/100 ml serum. The levels of sanguinarine found in the edible oils and the finding that pure argemone oil contained 5·4 mg sanguinarine/ml together indicated that the degree of adulteration of the edible oil samples with argemone oil ranged from 0·16 to 2·2%.

Introduction

There have been several outbreaks of epidemic dropsy in India since 1877 and various reports have dealt in detail with the epidemiological and clinical features of the disease. That epidemic dropsy is caused by the consumption of argemone oil present as an adulterant in mustard oil has been clearly documented (Lal & Das Gupta, 1941), and sanguinarine, the alkaloid present in argemone oil, has been incriminated as the causative agent for the disease (Sarkar, 1948). However, no information is so far available regarding the presence of sanguinarine in the circulation or urine of patients suffering from epidemic dropsy. This paper presents the results of analyses of samples of serum and urine obtained from patients severely affected with epidemic dropsy in the recent epidemic at Sirpur–Kagaznagar, Andhra Pradesh. Other details regarding the epidemic have been reported elsewhere (Krishnamachari & Satyanarayana, 1972).

Experimental

Materials. Paired serum and urine samples were obtained from 22 patients suffering from epidemic dropsy. Blood samples were taken under fasting conditions and the serum was separated. Random samples of urine were obtained, since the collection of 24-hr samples was impractical in the situation existing at the site of the epidemic. In addition, nine samples of edible oils were obtained from households in which there were patients suffering from epidemic dropsy, and three further oil samples were obtained from sealed tins obtained from different shops at Kagaznagar towards the end of the epidemic.

Analytical procedures. Sanguinarine was determined in all samples of oil, serum and urine, but the quantitative estimation of sanguinarine in the random urine samples was considered to have little significance.

Urine was concentrated to a convenient volume on a water bath, and all samples of urine and serum were deproteinized by the addition of trichloroacetic acid. An equal volume of conc. HCl was added to the supernatant solution, and the mixture was evaporated to dryness. The residue was extracted repeatedly with 1% acetic acid in chloroform and the solution was made up to a known volume. All or part of this chloroform extract was used for the detection and/or estimation of sanguinarine.

Sanguinarine was detected by the procedure of Chakravarti, Choudhuri, Das Gupta & Gita Datta (1959), suitably modified for thin-layer instead of paper chromatography and using the solvent system used by Hakim, Mijovic & Walker (1961). Quantitative determination of sanguinarine in the collected samples, and in samples of pure argemone oil expelled from the seeds in the laboratory, was carried out by the method of S. Babu, G. S. Thapar, I. S. Shenolikar & B. V. Rama Sastri (1972, to be published). For the oil analyses, 1 ml conc. HCl (AR), 0·5 ml ethanol and 2 ml of the oil sample in a stoppered test tube were shaken well for 2 min and then heated on a water-bath for 5–10 min. After filtration through a wet filter-paper, the filtrate was dried by evaporation on a water-bath and the residue was taken up in chloroform containing 1% glacial acetic acid. This chloroform solution and the chloroform extracts prepared from urine and sera as described above were spotted, along with pure standard sanguinarine, on thin-layer chromatographic plates and developed in butanol–acetic acid–water (63:10:27, by vol.). Plates were viewed under an ultraviolet lamp and the golden-yellow fluorescent band at R_F 0·45, which was in line with the standard sanguinarine spot, was cut out. A similar orange fluorescent spot (dihydrosanguinarine) at the solvent front was also cut out. The pooled silica powder was eluted with hot ethanol and made up to a known volume. The fluorescence was read in a spectrofluorimeter at excitation maximum 335 nm and emission maximum 490 nm. A standard curve was calibrated with standard sanguinarine and the levels in the unknown samples were read off from the standard curve.

Results and Discussion

The results of screening oil, urine and serum samples for the presence of sanguinarine are summarized in Table 1. All the oil samples obtained from households in which there were severe cases of dropsy contained detectable amounts of sanguinarine, although the samples obtained in sealed tins from various shops gave negative results. All the urine samples except one, but only two of the 22 serum samples contained detectable sanguinarine.

The extent of argemone oil adulteration in these edible oil samples was assessed from their sanguinarine content. For this purpose, a reference standard of a pure sample of argemone oil expressed in the laboratory from argemone seeds was used. Genuine argemone oil was found to contain 5·4 mg sanguinarine/ml. Based on this figure, the argemone oil content of the edible oil samples analysed in this investigation ranged between 0·16 and 2·20%. Since the exact amount of oil consumed by the patients suffering from epidemic dropsy could not be ascertained, no correlation between the consumption of the contaminated oil and the severity of the disease could be obtained. From the oil consumption pattern in the epidemic (Krishnamachari & Satyanarayana, 1972), it could be surmised that a daily intake of argemone oil as low as 0·02 ml or an intake of 108 μg sanguinarine could lead to the development of clinical manifestations. However, pure sanguinarine dissolved

Table 1. *Sanguinarine analyses of samples of edible oils and of serum and urine from patients with epidemic dropsy*

Material tested	Total no. of samples: Screened	Total no. of samples: Containing sanguinarine	Sanguinarine levels found (μg/100 ml)
Edible oil	9	9	864–11880
Serum	22	2*	4·7 & 28·3
Urine	22	21*	+ to + + + +†

*Limit of detection >0·5 μg/100 ml.
†Values in random urine samples ranging from approximately 0·5 μg/100 m. (+) to >2·0 μg/100 ml (+ + + +).

either in peanut oil or in ethylene glycol produced no signs of epidemic dropsy when injected into rats in a dose of 25 mg/kg body weight (equivalent to 5 ml argemone oil/kg) or given orally to monkeys in a dose of 1·6 mg/kg body weight (equivalent to 0·3 ml argemone oil/kg), although argemone oil containing similar amounts of sanguinarine did. This observation is in accordance with that reported by Lal. Das Gupta, Agarwala & Adak (1941) and suggests that in addition to the toxic alkaloid, sanguinarine, the presence of some other factor(s) in argemone oil is necessary to produce the disease (Rukmini, 1971). Identification of such factor(s) and the mode of action are now under investigation.

The two blood samples giving a positive result were found to contain 4·7 and 28·3 μg sanguinarine/100 ml serum and were obtained from two subjects who were severely affected. The other blood samples contained no detectable sanguinarine. However, the urine of all except one of the patients contained this alkaloid. Earlier it was observed that the minimum level of sanguinarine visually detectable by the procedure employed here was 10·8 ng/2 ml urine. Sanguinarine may therefore have been present at levels below the detectable 11 ng/2 ml in the serum samples from patients showing detectable levels in the urine. A lack of correlation between the presence of detectable levels of sanguinarine in the serum and high levels in the urine may have been due to the fact that urine analyses were limited to random samples. It is possible that some correlation might have been found if 24-hr urine samples could have been collected.

These data indicate that although no direct correlation between the serum levels of sanguinarine and the severity of epidemic dropsy could be shown, detection of sanguinarine in random samples of urine from affected persons could be used as a biochemical index to assess the adulteration of edible oils with argemone oil in communities where the disease occurs. It has been emphasized (Krishnamachari & Satyanarayana, 1972) that in this epidemic the adulteration of the edible oils with argemone oil was deliberate. This was clearly the case, since the oils consumed were peanut oil and sesame oil, the seeds of which do not resemble argemone seeds.

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REFERENCES

Chakravarti, R. N., Choudhuri, K. N., Das Gupta, B. & Gita Datta, A. (1959). Detection of traces of argemone oil in mustard oil, amino test. *J. Proc. Inst. Chem. Gt Br.* **31**, 118.

Hakim, S. A. E., Mijović, Valerie & Walker, J. (1961). Distribution of certain poppy-Fumaria alkaloids and a possible link with the incidence of glaucoma. *Nature, Lond.* **189**, 198.

Krishnamachari, K. A. V. R. & Satyanarayana, K. (1972). Epidemic dropsy in Andhra Pradesh. *Indian J. med. Res.* **60**, 741.

Lal, R. B. & Das Gupta, A. C. (1941). Investigations into the epidemiology of epidemic dropsy. Part X. A note on an outbreak of epidemic dropsy associated with the use of mustard oil pressed from the seeds adulterated. *Indian J. med. Res.* **29**, 157.

Lal, R. B., Das Gupta, A. C., Agarwala, S. P. & Adak, B. (1941). Investigations into the epidemiology of epidemic dropsy. Part XIII. Application of biological test to. *Indian J. med. Res.* **29**, 813.

Rukmini, C. (1971). Sanguinarine potentiating factor in argemone oil. *Indian J. med. Res.* **59**, 1676.

Sarkar, S. N. (1948). Isolation from argemone oil of dihydrosanguinarine and sanguinarine: Toxicity of sanguinarine. *Nature, Lond.* **162**, 265.

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MONOGRAPHS

MONOGRAPHS ON FRAGRANCE RAW MATERIALS*

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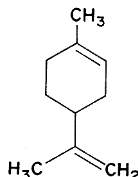
DIPENTENE

Synonyms: 1-Methyl-4-isopropenyl-1-cyclohexene; *d,l*-limonene; 1,8(9)-*p*-menthadiene.
Structure:



Description and physical properties: A colourless mobile liquid with a citrus-like odour (Arctander, 1969).
Occurrence: Found in a very large number of oils, including oils of lemongrass, citronella, palmarosa, cardamon and bergamot, Siberian pine needle oil and several other essential oils including turpentine oils of various origin (Guenther, 1949).
Preparation: As a by-product in the manufacture of terpineol and in various synthetic products made from α-pinene or turpentine oil (Arctander, 1969).
Uses: In public use since the 1940s. Use in fragrances in the USA amounts to less than 1000 lb/yr.

Concentration in final product (%):

	Soap	*Detergent*	*Creams, lotions*	*Perfume*
Usual	0·05	0·05		0·5
Maximum	0·75	0·075	0·25	2·0

Analytical data: Gas chromatogram, RIFM no. 72-21; infra-red curve, RIFM no. 72-21.

Status

Dipentene (*d,l*-limonene) was granted GRAS status by FEMA (1965) and is approved by the FDA as GRAS for food use. The Council of Europe (1970) included dipentene (*d,l*-limonene) in the list of admissible artificial flavouring substances, with a technological limit except for chewing gum.

*The most recent set of monographs in this series was published in *Food and Cosmetics Toxicology* 1974, **12**, 517.

Biological data

Acute toxicity. The acute oral LD_{50} value in rats was reported as 5·3 g/kg (4·6–6·0 g/kg) (Moreno, 1972a). The acute dermal LD_{50} value for *d*-limonene was reported as >5 g/kg (Moreno, 1972b).

Irritation. d-Limonene applied full strength to intact or abraded rabbit skin for 24 hr under occlusion was moderately irritating (Moreno, 1972b). Dipentene tested at 20% in petrolatum produced no irritation after a 48-hr closed-patch test in 25 human subjects (Kligman, 1972).

Sensitization. A maximization test (Kligman, 1966) was carried out on 25 volunteers. The material was tested at a concentration of 20% in petrolatum and produced no sensitization reactions (Kligman, 1972).

Additional published data

The autoxidation products of oil of turpentine (of Δ^3-carene) are eczematogenic (Pirilä, 1971).

References

Arctander, S. (1969). *Perfume and Flavor Chemicals (Aroma Chemicals).* Vol. I, p. 1074. S. Arctander, Montclair, New Jersey.
Council of Europe (1970). Natural and Artificial Flavouring Substances. Partial Agreement in the Social and Public Health Field. List A(1), Series 1, no. 493, p. 75. Strasbourg.
Flavoring Extract Manufacturers' Association (1965). Survey of flavoring ingredient usage levels. No. 2633. *Fd Technol., Champaign* **19** (2), part 2, 155.
Guenther, E. (1949). *The Essential Oils.* Vol. II, p. 27. C. Van Nostrand, Inc., Princeton, New Jersey.
Kligman, A. M. (1966). The identification of contact allergens by human assay. III. The maximization test. A procedure for screening and rating contact sensitizers. *J. invest. Derm.* **47**, 393.
Kligman, A. M. (1972). Report to RIFM, 25 August.
Moreno, O. M. (1972a). Report to RIFM, 5 May.
Moreno, O. M. (1972b). Report to RIFM, 31 May.
Pirilä, V. (1971). Pyhän Laurin tie IB, Helsinki 34 (Finland).

DIPHENYLMETHANE

Synonym: Benzylbenzene.
Structure: $C_6H_5 \cdot CH_2 \cdot C_6H_5$.
Description and physical properties: EOA Spec. no. 129.
Occurrence: Has apparently not been reported to occur in nature.
Preparation: By interaction of benzyl chloride and benzene in the presence of an acid catalyst.
Uses: In public use since the 1940s. Use in fragrances in the USA amounts to less than 4000 lb/yr.

Concentration in final product (%):

	Soap	*Detergent*	*Creams, lotions*	*Perfume*
Usual	0·03	0·005	0·01	0·05
Maximum	0·2	0·02	0·1	0·8

Analytical data: Gas chromatogram, RIFM no. 72-132; infra-red curve RIFM 72-132.

Status

The Council of Europe (1970) included diphenylmethane in the list of temporarily admissible artificial flavouring substances.

Biological data

Acute toxicity. The acute oral LD_{50} value in rats was reported as 2·25 g/kg (1·76–2·74 g/kg) (Moreno, 1973). The acute dermal LD_{50} value in rabbits was reported as >5 g/kg (Moreno, 1973).

Irritation. Diphenylmethane applied full strength to intact or abraded rabbit skin for 24 hr under occlusion was not irritating (Moreno, 1973). Tested at 8% in petrolatum it produced no irritation after a 48-hr closed-patch test in 25 human subjects (Kligman, 1973).

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

Metabolism. Diphenylmethane is hydroxylated in the rabbit and some 15% of the dose is excreted as 4-hydroxydiphenylmethane, which is largely (80–90%) in the free state. Neither the hydrocarbon nor its metabolite is oestrogenic. This reaction also occurs in the dog (Williams, 1959).

References

Council of Europe (1970). Natural and Artificial Flavouring Substances. Partial Agreement in the Social and Public Health Field. List A(1), Series 2, no. 121, p. 99. Strasbourg.
Kligman, A. M. (1966). The identification of contact allergens by human assay. III. The maximization test. A procedure for screening and rating contact sensitizers. *J. invest. Derm.* **47**, 393.
Kligman, A. M. (1973). Report to RIFM, 27 August.
Moreno, O. M. (1973). Report to RIFM, 1 February.
Williams, R. T. (1959). *Detoxication Mechanisms. The Metabolism and Detoxication of Drugs, Toxic Substances and Other Organic Compounds.* 2nd ed., p. 226. Chapman & Hall Ltd., London.

DIPHENYL OXIDE

Synonyms: Diphenyl ether; phenyl ether.
Structure: $C_6H_5 \cdot O \cdot C_6H_5$.
Description and physical properties: EOA Spec. no. 43.
Occurrence: Has apparently not been reported to occur in nature.
Preparation: By heating potassium phenolate with bromobenzene or with chlorobenzene at elevated temperatures (Bedoukian, 1967).
Uses: In public use since the 1930s. Use in fragrances in the USA amounts to about 100,000 lb/yr.

Concentration in final product (%):

	Soap	*Detergent*	*Creams, lotions*	*Perfume*
Usual	0·05	0·005	0·05	0·15
Maximum	0·20	0·03	0·10	0·40

Status

The Council of Europe (1970) included diphenyl oxide in the list of temporarily admissible artificial flavouring substances.

Biological data

Acute toxicity. The acute oral LD_{50} value in rats was reported as 3·37 g/kg (2·59–4·37 g/kg) (Weir, 1971). The acute dermal LD_{50} value in rabbits was reported as >5 g/kg (Weir, 1971).

Irritation. Diphenyl oxide applied full strength to intact or abraded rabbit skin for 24 hr under occlusion was slightly irritating (Weir, 1971). Applied to the eyes of rabbits, it produced negative results except for slight conjunctival irritation, which cleared in 72 hr (Weir, 1971).

Sensitization. A maximization test (Kligman, 1966) was carried out on 25 volunteers. The material was tested at a concentration of 4% in petrolatum and produced no sensitization reactions (Kligman, 1970).

References

Bedoukian, P. Z. (1967). *Perfumery and Flavoring Synthetics*. 2nd ed., p. 153. Elsevier Publishing Co., New York.
Council of Europe (1970). Natural and Artificial Flavouring Substances. Partial Agreement in the Social and Public Health Field. List A(1), Series 1, no. 215, p. 103. Strasbourg.
Kligman, A. M. (1966). The identification of contact allergens by human assay. III. The maximization test. A procedure for screening and rating contact sensitizers. *J. invest. Derm.* **47**, 393.
Kligman, A. M. (1970). Report to RIFM, 4 January.
Weir, R. J. (1971). Report to RIFM, 12 April.

ESTRAGON OIL

Synonym: Tarragon oil.
Description and physical properties: EOA Spec. no. 121. The chief component of estragon oil is methyl chavicol (Guenther, 1952).
Occurrence: Found in the plant *Artemesia dracunculus* L. (Fam. Compositae).
Preparation: By steam distillation of the leaves, stems and flowers of the plant *Artemesia dracunculus* L.
Uses: In public use since the 1920s. Use in fragrances in the USA amounts to less than 2000 lb/yr.

Concentration in final product (%):

	Soap	*Detergent*	*Creams, lotions*	*Perfume*
Usual	0·01	0·001	0·01	0·04
Maximum	0·1	0·01	0·1	0·4

Analytical data: Gas chromatogram, RIFM no. 72-134; infra-red curve, RIFM no. 72–134.

Status

Estragon oil was granted GRAS status by FEMA (1965) and is approved by the FDA as GRAS for food use. The Council of Europe (1970) included estragon oil (*Artemesia dracunculus*) in the list of substances, spices and seasonings whose use is deemed admissible with a possible limitation of the active principle in the final product. The *Food Chemicals Codex* (1972) has a monograph on estragon oil.

Biological data

Acute toxicity. The acute oral LD_{50} value in rats was reported as 1·9 ml/kg (1·5–2·5 ml/kg) (Shelanski, 1973a). The acute dermal LD_{50} in rabbits exceeded 5 ml/kg (Shelanski, 1973b).

Irritation. Estragon oil applied full strength to intact or abraded rabbit skin for 24 hr under occlusion was irritating (Shelanski, 1973b). It was also irritating when applied undiluted to the backs of hairless mice (Urbach & Forbes, 1973). Tested at 4% in petrolatum, it produced no irritation after a 48-hr closed-patch test in 25 human subjects (Kligman, 1972).

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

Phototoxicity. No phototoxic effects were reported for estragon oil (Urbach & Forbes, 1973).

References

Council of Europe (1970). Natural and Artificial Flavouring Substances. Part:al Agreement in the Social and Public Health Field. List N(1), Series 1(b), no. 64, p. 15. Strasbourg.

Flavoring Extract Manufacturers' Association (1965). Survey of flavoring ingredient usage levels. No. 2412. *Fd Technol., Champaign* **19** (2), part 2, 155.

Food Chemicals Codex (1972). 2nd ed. Prepared by the Committee on Specifications, Food Chemicals Codex, of the Committee on Food Protection. p. 807. National Academy of Sciences–National Research Council Publ. 1406, Washington, D.C.

Guenther, E. (1952). *The Essential Oils.* Vol. V. p. 463. D. Van Nostrand, Inc., Princeton, New Jersey.

Kligman, A. M. (1966). The identification of contact allergens by human assay. III. The maximization test. A procedure for screening and rating contact sensitizers. *J. invest. Derm.* **47**, 393.
Kligman, A. M. (1972). Report to RIFM, 1 November.
Shelanski, M. V. (1973a). Report to RIFM, 20 February.
Shelanski, M. V. (1973b). Report to RIFM, 30 January.
Urbach, F. & Forbes, P. D. (1973). Report to RIFM, 8 February.

ETHYL ACETATE

Synonym: Acetic ether.
Structure: $CH_3 \cdot CH_2 \cdot OCO \cdot CH_3$.
Description and physical properties: *Food Chemicals Codex* (1972).
Occurrence: Found in several essential oils (Gildemeister & Hoffman, 1966).
Preparation: From ethyl alcohol and acetic acid, usually in a multicolumnar distillation system (Arctander, 1969).
Uses: In public use since the 1920s. Use in fragrances in the USA amounts to less than 2000 lb/yr.

Concentration in final product (%):

	Soap	*Detergent*	*Creams, lotions*	*Perfume*
Usual	0·01	0·001	0·005	0·01
Maximum	0·05	0·005	0·05	1·0

Analytical data: Gas chromatogram, RIFM no. 71-6; infra-red curve, RIFM no. 71-6.

Status

Ethyl acetate was granted GRAS status by FEMA (1965) and is approved by the FDA as GRAS for food use. The Council of Europe (1970) listed ethyl acetate, giving an ADI of 2·5 mg/kg and the *Food Chemicals Codex* (1972) has a monograph on ethyl acetate. The *National Formulary* (1970) has a monograph on ethyl acetate and the Joint FAO/WHO Expert Committee on Food Additives (1967) has published a monograph and specifications for ethyl acetate giving an unconditional ADI of 0–25 mg/kg. Browning (1965) published a monograph on ethyl acetate.

Biological data

Acute toxicity. The acute oral LD_{50} in rats was reported as 5·6 g/kg while the inhalation LC_{50} in the rat was reported as 1600 ppm after 8 hr (Fassett, 1963).

Irritation. Ethyl acetate tested at 10% in petrolatum produced no irritation after a 48-hr closed-patch test in 25 human subjects (Kligman, 1972).

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

Metabolism. Ethyl acetate is hydrolysed to ethyl alcohol, which is then partly excreted in the expired air and urine. The rest is metabolized, the acetate fraction becoming incorporated in the body pool (Fassett, 1963).

Threshold limit value. The threshold limit value for ethyl acetate has been set at 400 ppm at which point it produces nose and throat irritation and has a mild narcotic action (American Conference of Governmental Industrial Hygienists, 1973).

Additional published data

Eyeglass dermatitis limited to the nose occurred in a 58-yr-old woman. Patch testing revealed the offending agent to be a common solvent, ethylene glycol monomethyl ether acetate, used to weld the nosepads chemically to the eyeglass frame. The patient also had an unrevealed allergy to ethyl acetate (Jordan & Dahl, 1971).

When ethyl acetate was injected into the yolk sac of fresh fertile chicken eggs before incubation in a dose of 9, 22·5, 45 or 90 mg/egg, the hatchabilities were 85, 50, 35 and 15% respectively (McLaughlin, Marliac, Verrett, Mutchler & Fitzhugh, 1964).

References

American Conference of Governmental Industrial Hygienists (1973). Threshold Limit Values for Chemical Substances and Physical Agents in the Workroom Environment. Cincinnati, Ohio.

Arctander, S. (1969). *Perfume and Flavor Chemicals (Aroma Chemicals)*. Vol. 1, p. 1137. S. Arctander, Montclair, New Jersey.

Browning, Ethel (1965). *Toxicity and Metabolism of Industrial Solvents*. p. 522. Elsevier Publishing Co., London.

Council of Europe (1970). Natural and Artificial Flavouring Substances. Partial Agreement in the Social and Public Health Field. List A(1), Series 1, no. 192, p. 59. Strasbourg.

Fassett, D. W. (1963). Esters. In *Industrial Hygiene and Toxicology*. 2nd ed. Edited by F. A. Patty. Vol. II, p. 1897. Interscience Publishers, New York.

Flavoring Extract Manufacturers' Association (1965). Survey of flavoring ingredient usage levels. No. 2414. *Fd Technol., Champaign* **19** (2), part 2, 155.

Food Chemicals Codex (1972). 2nd ed. Prepared by the Committee on Specifications, Food Chemicals Codex, of the Committee on Food Protection. p. 271. National Academy of Sciences–National Research Council Publ. 1406. Washington, D.C.

Gildemeister, E. u. Hoffman, F. (1966). *Die Ätherischen Öle*. Vol. IIId. Akademie Verlag, Berlin.

Joint FAO/WHO Expert Committee on Food Additives (1967). Toxicological Evaluation of Some Flavouring Substances and Non-nutritive Sweetening Agents. *F.A.O. Nutr. Mtg Rep. Ser. no.* 44A, Geneva, p. 23. WHO/Food Add./68.33.

Jordan, W. P., Jr. & Dahl, M. V. (1971). Contact dermatitis to a plastic solvent in eyeglasses. *Archs Derm.* **104**, 524.

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

Kligman, A. M. (1972). Report to RIFM, 2 May.

McLaughlin, J., Jr., Marliac, J.-P., Verrett, M. Jacqueline, Mutchler, Mary K. & Fitzhugh, O. G. (1964). Toxicity of fourteen volatile chemicals and measured by the chick embryo method. *Am. ind. Hyg. Ass. J.* **25**, 282.

National Formulary (1970). 13th ed. Prepared by the National Formulary Board. p. 297. American Pharmaceutical Association, Washington, D.C.

ETHYL ACETOACETATE

Synonyms: Acetoacetic ester; ethyl-3-oxobutanoate.
Structure: $CH_3 \cdot CO \cdot CH_2 \cdot OCO \cdot CH_2 \cdot CH_3$.
Description and physical properties: *Food Chemicals Codex* (1972).
Occurrence: Has apparently not been reported to occur in nature.
Preparation: From ethyl acetate by the action of sodium, sodium ethoxide, sodamide or calcium (*Merck Index*, 1968).
Uses: In public use since the 1950s. Use in fragrances in the USA amounts to less than 8000 lb/yr.

Concentration in final product (%):

	Soap	*Detergent*	*Creams, lotions*	*Perfume*
Usual	0·02	0·002	0·01	0·1
Maximum	0·1	0·01	0·10	0·8

Analytical data: Gas chromatogram, RIFM no. 72-135; infra-red curve, RIFM no. 72-135.

Status

Ethyl acetoacetate was granted GRAS status by FEMA (1965) and is approved by the FDA for food use (21 CFR 121.1164). The Council of Europe (1970) included ethyl acetoacetate in the list of admissible artificial flavouring substances, at a level of 500 ppm. The *Food Chemicals Codex* (1972) has a monograph on ethyl acetoacetate.

Biological data

Acute toxicity. The acute oral LD_{50} in rats was reported as 3·98 g/kg (Smyth, Carpenter & Weil, 1949). The acute dermal LD_{50} in rabbits was reported as > 5 g/kg (Moreno, 1973).

Irritation. Ethyl acetoacetate applied full strength to intact or abraded rabbit skin for 24 hr under occlusion was not irritating (Moreno, 1973). Tested at 8% in petrolatum, it produced no irritation after a 48-hr closed-patch test in 26 human subjects (Epstein, 1973).

Sensitization. A maximization test (Kligman, 1966) was carried out on 26 volunteers. The material was tested at a concentration of 8% in petrolatum and produced no sensitization reactions (Epstein, 1973).

References

Council of Europe (1970). Natural and Artificial Flavouring Substances. Partial Agreement in the Social and Public Health Field. List A(1), Series 1, no. 241, p. 62. Strasbourg.
Epstein, W. L. (1973). Report to RIFM, 17 July.
Flavoring Extract Manufacturers' Association (1965). Survey of flavoring ingredient usage levels. No. 2415. *Fd Technol., Champaign* **19** (2), part 2, 155.
Food Chemicals Codex (1972). 2nd ed. Prepared by the Committee on Specifications, Food Chemicals Codex, of the Committee on Food Protection, p. 272. National Academy of Sciences–National Research Council Publ. 1406, Washington, D.C.
Kligman, A. M. (1966). The identification of contact allergens by human assay. III. The maximization test. A procedure for screening and rating contact sensitizers. *J. invest. Derm.* **47**, 393.
Merck Index (1968). *An Encyclopedia of Chemicals and Drugs*. 8th ed., p. 430 Merck & Co., Inc., Rahway, New Jersey.
Moreno, O. M. (1973). Report to RIFM, 18 May.
Smyth, H. F., Jr., Carpenter, C. P. & Weil, C. S. (1949). Range-finding toxicity data, list III. *J. ind. Hyg. Toxicol.* **31**, 60.

ETHYL AMYL KETONE

Synonym: 3-Octanone.
Structure: $CH_3 \cdot [CH_2]_4 \cdot CO \cdot CH_2 \cdot CH_3$.
Description and physical properties: *Givaudan Index* (1961).
Occurrence: Found in oil of lavender (*Givaudan Index*, 1961).
Preparation: By heating propionic and caproic acids over thorium oxide or by oxidation of ethyl amyl carbinol (3-octanol) (Arctander, 1969).
Uses: In public use since the 1930s. Use in fragrances in the USA amounts to less than 2000 lb/yr.

Concentrations in final product (%):

	Soap	*Detergent*	*Creams, lotions*	*Perfume*
Usual	0·01	0·001	0·01	0·04
Maximum	0·05	0·02	0·05	0·2

Analytical data: Gas chromatogram, RIFM no. 72-136; infra-red curve, RIFM no. 72-136.

Status

Ethyl amyl ketone was granted GRAS status by FEMA (1965) and is approved by the FDA for food use (21 CFR 121.1164). The Council of Europe (1970) included ethyl amyl ketone in the list of temporarily admissible artificial flavouring substances.

Biological data

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

Irritation. Ethyl amyl ketone applied full strength to intact or abraded rabbit skin for 24 hr under occlusion was moderately irritating (Shelanski, 1973). Tested at 2% in petrolatum, it produced no irritation after a 48-hr closed-patch test in 25 human subjects (Kligman, 1972).

Sensitization. A maximization test (Kligman, 1966) was carried out on 25 volunteers. The material was tested at a concentration of 2% in petrolatum and produced no sensitization reactions (Kligman, 1972).

References

Arctander, S. (1969). *Perfume and Flavor Chemicals (Aroma Chemicals)*. Vol. 1, p. 1148. S. Arctander, Montclair, New Jersey.

Council of Europe (1970). Natural and Artificial Flavouring Substances. Partial Agreement in the Social and Public Health Field. List A(1), Series 2, no. 44, p. 96. Strasbourg.

Flavoring Extract Manufacturers' Association (1965). Survey of flavoring ingredient usage levels. No. 2803. *Fd Technol., Champaign* **19** (2), part 2, 155.

Givaudan Index (1961). *Specifications of Synthetics and Isolates for Perfumery*. 2nd ed., p. 144. Givaudan-Delawanna, Inc., New York.

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

Kligman, A. M. (1972). Report to RIFM, 1 November.

Shelanski, M. V. (1973). Report to RIFM, 30 January.

ETHYL BENZOATE

Structure: $CH_3 \cdot CH_2 \cdot OCO \cdot C_6H_5$.
Description and physical properties: EOA Spec. no. 210.
Occurrence: Has apparently not been reported to occur in nature.
Preparation: By esterification of ethyl alcohol and benzoic acid.
Uses: In public use since the 1940s. Use in fragrances in the USA amounts to less than 5000 lb/yr.

Concentration in final product (%):

	Soap	*Detergent*	*Creams, lotions*	*Perfume*
Usual	0·01	0·003	0·01	0·02
Maximum	0·1	0·01	0·1	0·8

Analytical data: Infra-red curve, RIFM no. 72-137.

Status

Ethyl benzoate was granted GRAS status by FEMA (1965) and is approved by the FDA for food use (21 CFR 121.1164). The Council of Europe (1970) listed ethyl benzoate, giving an ADI of 5 mg/kg. The *Food Chemicals Codex* (1972) has a monograph on ethyl benzoate and Browning (1965) has published an extensive monograph on this ester.

Biological data

Acute toxicity. The acute oral LD_{50} value in rats was reported as 6·480 g/kg (Bär & Griepentrog, 1967). The acute dermal LD_{50} value in rabbits was reported as 1·94 g/kg (Graham & Kuizenga, 1945).

Irritation. Ethyl benzoate tested at 8% in petrolatum produced no irritation after a 48-hr closed-patch test in 25 human subjects (Kligman, 1972).

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

References

Bär, F. u. Griepentrog, F. (1967). Die Situation in der gesundheitlichen Beurteilung der Aromatisierungsmittel für Lebensmittel. *Medizin Ernähr.* **8**, 144.

Browning, Ethel (1965). *Toxicity and Metabolism of Industrial Solvents*. p. 589. Elsevier Publishing Co., London.

Council of Europe (1970). Natural and Artifical Flavouring Substances. Partial Agreement in the Social and Public Health Field. List A(1), Series 1, no. 262. p. 63. Strasbourg.

Flavoring Extract Manufacturers' Association (1965). Survey of flavoring ingredient usage levels. No. 2422. *Fd Technol., Champaign* **19** (2), part 2, 155.

Food Chemicals Codex (1972). 2nd ed. Prepared by the Committee on Specifications, Food Chemicals Codex, of the Committee on Food Protection. p. 281. National Academy of Sciences–National Research Council Publ. 1406, Washington, D.C.

Graham, B. E. & Kuizenga, M. H. (1945). Toxicity studies on benzyl benzoate and related benzyl compounds. *J. Pharm. exp. Ther.* **84**, 358.

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

Kligman, A. M. (1972). Report to RIFM, 13 October.

ETHYL BUTYRATE

Synonyms: Ethyl *n*-butanoate; butyric ether.
Structure: $CH_3 \cdot CH_2 \cdot OCO \cdot [CH_2]_2 \cdot CH_3$.
Description and physical properties: EOA Spec. no. 108.
Occurrence: Reported to occur in several oils (Gildemeister & Hoffman, 1966).
Preparation: By the esterification of normal butyric acid with ethyl alcohol.
Uses: In public use since the late 1800s. Use in fragrances in the USA amounts to less than 51,000 lb/yr.

Concentration in final product (%):

	Soap	*Detergent*	*Creams, lotions*	*Perfume*
Usual	0·005	—	0·005	0·01
Maximum	0·05	0·001	0·05	0·5

Analytical data: Gas chromatogram, RIFM no. 71-46; infra-red curve, RIFM no. 71-46.

Status

Ethyl butyrate was granted GRAS status by FEMA (1965) and is approved as GRAS by the FDA for food use. The Council of Europe (1970) listed ethyl butyrate, giving an ADI of 1 mg/kg. The *Food Chemicals Codex* (1972) has a monograph on ethyl butyrate. The Joint FAO/WHO Expert Committee on Food Additives (1967) has published a monograph and specifications for ethyl butyrate giving an unconditional ADI of 0–15 mg/kg, and Browning (1965) has also published a monograph on ethyl butyrate.

Biological data

Acute toxicity. The acute oral LD_{50} value in rats was reported as 13 g/kg (Jenner, Hagan, Taylor, Cook & Fitzhugh, 1964). The acute dermal LD_{50} value in rabbits was reported as >2 g/kg (Moreno, 1972).

Irritation. Ethyl butyrate applied full strength to intact or abraded rabbit skin for 24 hr under occlusion was moderately irritating (Moreno, 1972). Tested at 5% in petrolatum, it produced no irritation after a 48-hr closed-patch test in 25 human subjects (Kligman, 1972).

Sensitization. A maximization test (Kligman, 1966) was carried out on 25 volunteers. The material was tested at a concentration of 5% in petrolatum and produced no sensitization reactions (Kligman, 1972).

References

Browning, Ethel (1965). *Toxicity and Metabolism of Industrial Solvents*. p. 579. Elsevier Publishing Co., London.
Council of Europe (1970). Natural and Artificial Flavouring Substances. Partial Agreement in the Social and Public Health Field. List A(1), Series 1, no. 265, p. 63. Strasbourg.
Flavoring Extract Manufacturers' Association (1965). Survey of flavoring ingredient usage levels. No. 2427. *Fd Technol., Champaign* **19** (2), part 2, 155.
Food Chemicals Codex (1972). 2nd ed. Prepared by the Committee on Specifications, Food Chemicals Codex, of the Committee on Food Protection, p. 283. National Academy of Sciences–National Research Council Publ. 1406. Washington, D.C.
Gildemeister, E. u. Hoffman, F. (1966). *Die Ätherischen Öle*. Vol. IIId, p. 197. Akademie Verlag, Berlin.
Jenner, P. M., Hagan, E. C., Taylor, Jean M., Cook, E. L. & Fitzhugh, O. G. (1964). Food flavourings and compounds of related structure. I. Acute oral toxicity. *Fd Cosmet. Toxicol.* **2**, 327.

Joint FAO/WHO Expert Committee on Food Additives (1967). Toxicological Evaluation of Some Flavouring Substances and Non-nutritive Sweetening Agents. *F.A.O. Nutr. Mtg Rep. Ser. no.* 44A, Geneva, p. 25. WHO/Food Add./68.33.

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

Kligman, A. M. (1972). Report to RIFM, 2 May.

Moreno, O. M. (1972). Report to RIFM, 19 February.

ETHYL CINNAMATE

Synonyms: Ethyl β-phenylacrylate; ethyl 3-phenylpropenoate; ethyl *trans*-cinnamate.
Structure: $C_6H_5 \cdot CH{:}CH \cdot OCO \cdot CH_2 \cdot CH_3$.
Description and physical properties: *The Givaudan Index* (1961).
Occurrence: Found in storax oil, *Kaempferia galanga* and several other oils (Gildemeister & Hoffman, 1966).
Preparation: By direct esterification of ethanol with cinnamic acid under azeotropic conditions or by Claisen-type condensation of ethyl acetate and benzaldehyde in the presence of sodium metal (Bedoukian, 1967).
Uses: In public use since the 1930s. Use in fragrances in the USA amounts to less than 1000 lb/yr.

Concentration in final product (%):

	Soap	*Detergent*	*Creams, lotions*	*Perfume*
Usual	0·02	0·002	0·02	0·08
Maximum	0·1	0·01	0·1	0·4

Status

Ethyl cinnamate was granted GRAS status by FEMA (1965) and is approved by the FDA for food use (21 CFR 121.1164). The Council of Europe (1970) listed ethyl cinnamate, giving an ADI of 1·25 mg/kg. The *Food Chemicals Codex* (1972) has a monograph on ethyl cinnamate.

Biological data

Acute toxicity. The acute oral LD_{50} value in rats was reported as 7·8 g/kg (7·41–8·19 g/kg) (Russell, 1973). The acute dermal LD_{50} value in rabbits was reported as >5 g/kg (Russell, 1973).

Irritation. Ethyl cinnamate applied full strength to intact or abraded rabbit skin for 24 hr under occlusion was not irritating (Russell, 1973). Tested at 4% in petrolatum, it produced no irritation after a 48-hr closed-patch test in 25 human subjects (Kligman, 1973). A patch test using ethyl cinnamate at full strength for 24 hr produced one out of 22 irritation reactions (Katz, 1946). Tested on the forearms of volunteers, it was found to be free of irritating properties (Peterson & Hall, 1946).

Sensitization. A maximization test (Kligman, 1966) was carried out on 25 volunteers. The material was tested at a concentration of 4% in petrolatum and produced no sensitization reactions (Kligman, 1973).

References

Bedoukian, P. Z. (1967). *Perfumery and Flavoring Synthetics*. 2nd ed., p. 82. Elsevier Publishing Co., New York.
Council of Europe (1970). Natural and Artificial Flavouring Substances. Partial Agreement in the Social and Public Health Field. List A(1), Series 1, no. 324, p. 66. Strasbourg.
Flavoring Extract Manufacturers' Association (1965). Survey of flavoring ingredient usage levels. No. 2430. *Fd Technol., Champaign* **19** (2), part 2, 155.
Food Chemicals Codex (1972). 2nd ed. Prepared by the Committee on Specifications, Food Chemicals Codex, of the Committee on Food Protection. p. 286. National Academy of Sciences–National Research Council Publ. 1406, Washington, D.C.
Gildemeister, E. u. Hoffman, F. (1966). *Die Ätherischen Öle*. Vol. IIId, p. 339. Akademie Verlag, Berlin.
Givaudan Index (1961). *Specifications of Synethetics and Isolates for Perfumery*. 2nd ed., p. 148. Givaudan-Delawanna, Inc., New York.

Katz, A. (1946). *Spice Mill* **69** (July), 46.
Kligman, A. M. (1966). The identification of contact allergens by human assay. III. The maximization test. A procedure for screening and rating contact sensitizers. *J. invest. Derm.* **47**, 393.
Kligman, A. M. (1973). Report to RIFM, 27 July.
Peterson, H. R. & Hall, A. (1946). Dermal irritating properties of perfume materials. *Drug Cosmet. Ind.* **58**, 113.
Russell, T. J. (1973). Report to RIFM, 6 March.

GRAPEFRUIT OIL EXPRESSED

Description and physical properties: EOA Spec. no. 30. The main constituent of grapefruit oil expressed is limonene (Gildemeister & Hoffman, 1959; Guenther, 1949).
Occurrence: Found in the fresh peel of the fruit *Citrus paradisi* Macfayden (Fam. Rutaceae).
Preparation: By expression of the fresh peel of the fruit.
Uses: In public use since the 1930s. Use in fragrances in the USA amounts to less than 18,000 lb/yr.

Concentration in final product (%):

	Soap	*Detergent*	*Creams, lotions*	*Perfume*
Usual	0·05	0·005	0·03	0·5
Maximum	0·3	0·03	0·15	1·0

Analytical data: Gas chromatogram, RIFM nos 72-24 and 73-21; infra-red curve, RIFM nos 72-24 and 73-21.

Status

Grapefruit oil was granted GRAS status by FEMA (1965) and is approved by the FDA as GRAS for food use. The Council of Europe (1970) included grapefruit oil in the list of fruits and vegetables or parts thereof, parts for which no restriction is proposed. The *Food Chemicals Codex* (1972) has a monograph on grapefruit oil.

Biological data

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

Irritation. Undiluted grapefruit oil applied to the backs of hairless mice was not irritating (Urbach & Forbes, 1972). Applied full strength to intact or abraded rabbit skin for 24 hr under occlusion it was slightly irritating (Moreno, 1973), and tested at 10% in petrolatum, it produced no irritation after a 48-hr closed-patch test in 24 human subjects (Kligman, 1971). A patch test using full strength grapefruit oil for 24 hr produced no reactions in 26 subjects (Katz, 1946).

Sensitization. A maximization test (Kligman, 1966) was carried out on 24 volunteers. The material was tested at a concentration of 10% in petrolatum and produced no sensitization reactions (Kligman, 1971). A repeated-insult patch test using a 4% concentration in petrolatum did not sensitize any of 40 subjects (Thomas, 1971).

Phototoxicity. No phototoxic effects were reported for grapefruit oil (Urbach & Forbes, 1972).

Additional published data

The presence and identity of terpenes, psoralens and coumarins in grapefruit oil has been reported (Kesterson, Hendrickson & Braddock, 1971). Antibacterial properties have been reported for grapefruit oil (Dabbah, Edwards & Moats, 1970), and the oil has been reported to promote tumour formation on the skins of mice treated with the primary carcinogen 7,12-dimethylbenz[*a*]anthracene (Roe & Field, 1965).

References

Council of Europe (1970). Natural and Artificial Flavouring Substances. Partial Agreement in the Social and Public Health Field. List N(1), Series 1(a), no. 140, p. 18. Strasbourg.

Dabbah, R., Edwards, V. M. & Moats, W. A. (1970). Antimicrobial action of some citrus fruit oils on selected food-borne bacteria. *Appl. Microbiol.* **19**, 27.

Flavoring Extract Manufacturers' Association (1965). Survey of flavoring ingredient usage levels. No. 2530. *Fd Technol., Champaign* **19** (2), part 2, 155.

Food Chemicals Codex (1972). 2nd ed. Prepared by the Committee on Specifications, Food Chemicals Codex, of the Committee on Food Protection. p. 360. National Academy of Sciences-National Research Council Publ. 1406, Washington, D.C.

Gildemeister, E. u. Hoffman, F. (1959). *Die Ätherischen Öle*. Vol. V. p. 603. Akademie Verlag, Berlin.

Guenther, E. (1949). *The Essential Oils*. Vol. III. p. 347. D. Van Nostrand, Inc., Princeton, New Jersey.

Katz, A. (1946). *Spice Mill* **69** (July), 46.

Kesterson, J. W., Hendrickson, R. & Braddock, R. J. (1971). Florida Citrus Oils. Agricultural Experiment Stations. Bulletin 749 (technical), University of Florida, Gainesville.

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

Kligman, A. M. (1971). Report to RIFM, 23 March.

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

Roe, F. J. C. & Field, Winifred E. H. (1965). Chronic toxicity of essential oils and certain other products of natural origin. *Fd Cosmet. Toxicol.* **3**, 311.

Thomas, M. J. (1971). Report to RIFM, 13 April.

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

LEMON OIL EXPRESSED

Description and physical properties: *Food Chemicals Codex* (1972). The main constituent of lemon oil is *d*-limonene (Gildemeister & Hoffman, 1959; Guenther, 1949).
Occurrence: Found in the peel of the fruit *Citrus limon* (Linn.) Burm. F., (Fam. Rutaceae) (Guenther, 1949).
Preparation: By expression of the ripe peel of the fruit (Arctander, 1960).
Uses: In public use since the early 1800s. Use in fragrances in the USA amounts to about 150,000 lb/yr.

Concentration in final product (%):

	Soap	*Detergent*	*Creams, lotions*	*Perfume*
Usual	0·05	0·005	0·25	0·50
Maximum	0·4	0·004	0·15	1·0

Analytical data: Gas chromatogram, RIFM nos 72-32 and 72-61; infra-red curve, RIFM nos 72-32 and 72-61.

Status

Lemon oil was granted GRAS status by FEMA (1965) and is approved by the FDA as GRAS for food use. The Council of Europe (1970) included lemon oil in the list of substances, spices and seasonings whose use is deemed admissible, with a possible limitation of the active principle in the final product. The *Food Chemicals Codex* (1972) and the *United States Pharmacopeia* (1965) have monographs on lemon oil.

Biological data

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

Irritation. Lemon oil applied full strength to intact or abraded rabbit skin for 24 hr under occlusion was moderately irritating (Hart, 1971). Three samples of lemon oil (RIFM nos 72-32, 72-61 and 72–241) applied undiluted to the backs of hairless mice were mildly irritating (Urbach & Forbes, 1972), but three other samples of lemon oil (RIFM nos 72-249, 72-230 and 72–251) similarly applied undiluted to the backs of hairless mice were not irritating (Urbach & Forbes, 1972). Lemon oil tested at 10% in petrolatum produced no irritation after a 48-hr closed-patch test in 25 human subjects (Kligman, 1971). A patch test using full strength lemon oil for 24 hr produced no reactions in 24 human subjects (Katz, 1946).

Sensitization. A maximization test (Kligman, 1966) was carried out on 25 volunteers. The material was tested at a concentration of 10% in petrolatum and produced no sensitization reactions (Kligman, 1971).

Phototoxicity. Distinct phototoxic effects were reported by Urbach & Forbes (1972) for five samples of lemon oil—RIFM nos 72-61, 72-249, 72-250 (Italian), 72-241 (Greek) and 72-251 (Ivory Coast). Low-level phototoxic effects were reported for lemon oil (California; RIFM no. 72-32) (Urbach & Forbes, 1972).

Additional published data

The presence and identity of coumarins and psoralens in lemon oil has been reported (Kesterson, Hendrickson & Braddock, 1971; Stanley & Vannier, 1957). Antibacterial

properties have been reported for lemon oil (Dabbah, Edwards & Moats, 1970), which has also been reported to promote tumour formation on the skins of mice treated with the primary carcinogen, 7,12-dimethylbenz[*a*]anthracene (Roe & Field, 1965).

References

Arctander, S. (1960). *Perfume and Flavor Materials of Natural Origin*. p. 354. S. Arctander, Elizabeth, New Jersey.

Council of Europe (1970). Natural and Artificial Flavouring Substances. Partial Agreement in the Social and Public Health Field. List N(1), Series 1(b), no. 139, p. 17. Strasbourg.

Dabbah, R., Edwards, V. M. & Moats, W. A. (1970). Antimicrobial action of some citrus fruit oils on selected food-borne bacteria. *Appl. Microbiol.* **19**, 27.

Flavoring Extract Manufacturers' Association (1965). Survey of flavoring ingredient usage levels. No. 2625. *Fd Technol., Champaign* **19** (2), part 2, 155.

Food Chemicals Codex (1972). 2nd ed. Prepared by the Committee on Specifications, Food Chemicals Codex, of the Committee on Food Protection. p. 448. National Academy of Sciences–National Research Council Publ. 1406, Washington, D.C.

Gildemeister, E. u. Hoffman, F. (1959). *Die Ätherischen Öle*. Vol. V. p. 488. Akademie Verlag, Berlin.

Guenther, E. (1949). *The Essential Oils*. Vol. III. p. 81. D. Van Nostrand, Inc., Princeton, New Jersey.

Hart, E. R. (1971). Report to RIFM. 30 July.

Katz, A. (1946). *Spice Mill* **69** (July), 46.

Kesterson, J. W., Hendrickson, R. & Braddock, R. J. (1971). Florida Citrus Oils. Agricultural Experiment Stations. Bulletin 749 (technical). University of Florida, Gainesville.

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

Kligman, A. M. (1971). Report to RIFM. 20 April.

Roe, F. J. C. & Field, Winifred E. H. (1965). Chronic toxicity of essential oils and certain other products of natural origin. *Fd Cosmet. Toxicol.* **3**, 311.

Stanley, W. L. & Vannier, S. H. (1957). Chemical composition of lemon oil. I. Isolation of a series of substituted coumarins. *J. Am. chem. Soc.* **79**, 3488.

United States Pharmacopeia (1965). 17th revision. Prepared by the Committee of Revision. p. 337. The United States Pharmacopeial Convention, Inc., New York.

Urbach, F. & Forbes, P. D. (1972). Report to RIFM. 26 May; 22 September and 19 December.

LEMON OIL DISTILLED*

Preparation: By steam distillation of chopped lemon peel under partial vacuum (Arctander, 1960).

Biological data

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

Irritation. Lemon oil applied full strength to intact or abraded rabbit skin for 24 hr under occlusion was moderately irritating (Hart, 1971). Lemon oil distilled (RIFM no. 72-240) applied undiluted to the backs of hairless mice was slightly irritating (Urbach & Forbes, 1972). Lemon oil FCF applied undiluted to the backs of hairless mice was not irritating (Urbach & Forbes, 1974). Lemon oil tested at 10% in petrolatum produced no irritation after a 48-hr closed-patch test in 25 human subjects (Kligman, 1971).

Sensitization. A maximization test (Kligman, 1966) was carried out on 25 volunteers. The material was tested at a concentration of 10% in petrolatum and produced no sensitization reactions (Kligman, 1971).

Phototoxicity. No phototoxic effects were reported for lemon oil distilled, RIFM no. 72-240 (Urbach & Forbes, 1972) or for lemon oil FCF (Urbach & Forbes, 1974).

References

Arctander, S. (1960). *Perfume and Flavor Materials of Natural Origin*. p. 354. S. Arctander, Elizabeth, New Jersey.
Hart, E. R. (1971). Report to RIFM, 30 July.
Kligman, A. M. (1966). The identification of contact allergens by human assay. III. The maximization test. A procedure for screening and rating contact sensitizers. *J. invest. Derm.* **47**, 393.
Kligman, A. M. (1971). Report to RIFM, 20 April.
Urbach, F. & Forbes, P. D. (1972). Report to RIFM, 22 September.
Urbach, F. & Forbes, P. D. (1974). Report to RIFM, 15 March.

*See also preceding monograph on Lemon Oil Expressed.

LIME OIL DISTILLED

Description and physical properties: EOA Spec. no. 78. The main constituent of lime oil distilled is *d*-limonene (Gildemeister & Hoffman, 1959; Guenther, 1949).
Occurrence: Found in the fruit *Citrus aurantifolia* Swingle formerly classified as *Citrus medica* L., var acida Brandis (Fam. Rutaceae).
Preparation: By distillation of the whole crushed fruit.
Uses: In public use before the 1900s. Use in fragrances in the USA amounts to about 50,000 lb/yr.

Concentration in final product (%):

	Soap	*Detergent*	*Creams, lotions*	*Perfume*
Usual	0·05	0·005	0·03	0·4
Maximum	0·25	0·025	0·1	1·5

Analytical data: Gas chromatogram, RIFM no. S-2276.

Status

Lime oil was granted GRAS status by FEMA (1965) and is approved as GRAS by the FDA for food use. The Council of Europe (1970) included lime oil in the list of substances, spices and seasonings whose use is deemed admissible, with a possible limitation of the active principle in the final product. The *Food Chemicals Codex* (1972) has a monograph on lime oil distilled.

Biological data

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

Irritation. Lime oil applied full strength to intact or abraded rabbit skin for 24 hr under occlusion was slightly irritating (Hart, 1971). Tested at 15% in petrolatum, it produced no irritation after a 48-hr closed-patch test in 25 human subjects (Kligman, 1971). A patch test using full strength lime oil for 24 hr produced no reactions in 20 subjects (Katz, 1946).

Sensitization. A maximization test (Kligman, 1966) was carried out on 25 volunteers. The material was tested at a concentration of 15% in petrolatum and produced no sensitization reactions (Kligman, 1971).

Phototoxicity. No phototoxic effects were reported for lime oil distilled (Urbach & Forbes, 1972).

References

Council of Europe (1970). Natural and Artificial Flavouring Substances. Partial Agreement in the Social and Public Health Field. List N(1), Series 1(b), no. 141, p. 18. Strasbourg.
Flavoring Extract Manufacturers' Association (1965). Survey of flavoring ingredient usage levels. No. 2631. *Fd Technol., Champaign* **19** (2), part 2, 155.
Food Chemicals Codex (1972). 2nd ed. Prepared by the Committee on Specifications, Food Chemicals Codex of the Committee on Food Protection. p. 453. National Academy of Sciences–National Research Council Publ. 1406, Washington, D.C.
Gildemeister, E. u. Hoffman, F. (1959). *Die Ätherischen Öle*. Vol. V, p. 583. Akademie Verlag, Berlin.
Guenther, E. (1949). *The Essential Oils*. Vol. III, p. 325. D. Van Nostrand, Inc., Princeton, New Jersey.
Hart, E. R. (1971). Report to RIFM, 18 June.
Katz, A. (1946). *Spice Mill* **69** (July), 46.
Kligman, A. M. (1966). The identification of contact allergens by human assay. III. The maximization test. A procedure for screening and rating contact sensitizers. *J. invest. Derm.* **47**, 393.
Kligman, A. M. (1971). Report to RIFM, 9 June.
Urbach, F. & Forbes, P. D. (1972). Report to RIFM, 23 March.

LIME OIL EXPRESSED

Description and physical properties: EOA Spec. no. 88. The chief constituent of lime oil expressed is *d*-limonene (Gildemeister & Hoffman, 1959; Guenther, 1949).
Occurrence: Found in the peel of the fruit *Citrus aurantifolia* Swingle, formerly classified as *Citrus medica* L., var acida Brandis (Fam. Rutaceae).
Preparation: By expression from the fresh peel of the fruit.
Uses: In public use since the 1800s. Use in fragrances in the USA amounts to about 50,000 lb/yr.

Concentration in final product (%):

	Soap	*Detergent*	*Creams, lotions*	*Perfume*
Usual	0·05	0·005	0·03	0·5
Maximum	0·25	0·025	0·1	1·5

Status

Lime oil was granted GRAS status by FEMA (1965) and is approved by the FDA as GRAS for food use. The Council of Europe (1970) included lime oil in the list of substances, spices and seasonings whose use is deemed admissible, with a possible limitation of the active principle in the final product.

Biological data*

Phototoxicity. Lime oil expressed was found to have phototoxic properties when tested on hairless mice, pigs and man (Urbach & Forbes, 1972).

Additional published data

Lime oil has been reported as a tumour promoter on the skin and in the forestomach epithelium of mice treated with the primary carcinogen, 7,12-dimethylbenz[*a*]anthracene (Roe & Field, 1965). Eleven cases of photodermatitis due to oil of Persian lime have been reported and a photodynamic reaction was experimentally produced by oil from the Persian lime on the skin and subsequent solar irradiation (Sams, 1941). The presence and identity of coumarins and psoralens in lime oil has been reported (Kesterson, Hendrickson & Braddock, 1971; Stanley & Vannier, 1967).

References

Council of Europe (1970). Natural and Artificial Flavouring Substances. Partial Agreement in the Social and Public Health Field. List N(1). Series 1(b). no. 141. p. 18. Strasbourg.

Flavoring Extract Manufacturers' Association (1965). Survey of flavoring ingredient usage levels. No. 2631. *Fd Technol., Champaign* **19** (2), part 2, 155.

Gildemeister, E. u. Hoffman, F. (1959). *Die Ätherischen Öle*. Vol. V. p. 571. Akademie Verlag, Berlin.

Guenther, E. (1949). *The Essential Oils*. Vol. III. p. 324. D. Van Nostrand, Inc., Princeton, New Jersey.

Kesterson, J. W., Hendrickson, R. & Braddock, R. J. (1971). Florida Citrus Oils. Agricultural Experiment Stations. Bulletin 749 (technical). University of Florida, Gainesville.

Roe, F. J. C. & Field, Winifred E. H. (1965). Chronic toxicity of essential oils and certain other products of natural origin. *Fd Cosmet. Toxicol.* **3**, 311.

Sams, W. M. (1941). Photodynamic action of lime oil (*Citrus aurantifolia*). *Archs Derm. Syph.* **44**, 571.

Stanley, W. L. & Vannier, S. H. (1967). Psoralens and substituted coumarins from expressed oil of lime. *Phytochemistry* **6**, 585.

Urbach, F. & Forbes, P. D. (1972). Report to RIFM, 23 March.

*See also preceding monograph on Lime Oil Distilled.

ORANGE OIL EXPRESSED

Description and physical properties: *Food Chemicals Codex* (1972). The main constituent of orange oil is limonene (Gildemeister & Hoffman, 1959; Guenther, 1949).
Occurrence: Found in the peel of the fruit *Citrus sinensis* (Linn.) Osbeck (Fam. Rutaceae) (Gildemeister & Hoffman, 1959; Guenther, 1949).
Preparation: By expression of the peel of the ripe fruit (Arctander, 1960).
Uses: In public use since the 1800s. Use in fragrances in the USA amounts to about 200,000 lb/yr.

Concentration in final product (%):

	Soap	*Detergent*	*Creams, lotions*	*Perfume*
Usual	0·05	0·005	0·03	0·5
Maximum	0·4	0·04	0·1	0·80

Analytical data: Gas chromatogram, RIFM nos 71-63, 72-43 and 73-30; infra-red curve, RIFM nos 71-63, 72-43 and 73-30.

Status

Orange oil was granted GRAS status by FEMA (1965) and is approved by the FDA as GRAS for food use. The Council of Europe (1970) included orange oil in the list of substances, spices and seasonings whose use is deemed admissible, with a possible limitation of the active principle in the final product. The *Food Chemicals Codex* (1972) and the *United States Pharmacopeia* (1965) both have monographs on orange oil.

Biological data

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

Irritation. Orange oil undiluted applied to the backs of hairless mice was not irritating (Urbach & Forbes, 1972). Applied full strength to intact or abraded rabbit skin for 24 hr under occlusion it was moderately irritating (Moreno, 1973). Orange oil tested at 8% in petrolatum produced no irritation after a 48-hr closed-patch test in 21 human subjects (Epstein, 1973), and a patch test using orange oil full strength for 24 hr produced no reactions in 25 subjects (Katz, 1946).

Sensitization. A maximization test (Kligman, 1966) was carried out on 21 volunteers. The material was tested at a concentration of 8% in petrolatum and produced no sensitization reactions (Epstein, 1973).

Phototoxicity. No phototoxic effects were reported for orange oil (Urbach & Forbes, 1972).

Additional published data

Orange oil has been reported to promote tumour formation on the skins of mice treated with the primary carcinogen, 7,12-dimethylbenz[*a*]anthracene (Roe & Field, 1965). While orange oil and various terpenes have been described as co-carcinogens (Pierce, 1959; Roe, 1959), it appears that a terpene, namely *d*-limonene, also exerts anticarcinogenic or chemoprophylactic effects against the carcinogenic effects of sc injected benzo[*r,s,t*]pentaphene (Homburger, Treger & Boger, 1971).

Antibacterial properties have been reported for orange oil (Dabbah, Edwards & Moats, 1970).

The presence and identity of terpenes, paraffin waxes and α,β-dialkyl acroleins in orange oil have been reported (Kesterson, Hendrickson & Braddock, 1971).

References

Arctander, S. (1960). *Perfume and Flavor Materials of Natural Origin.* p. 484. S. Arctander, Elizabeth, New Jersey.

Council of Europe (1970). Natural and Artificial Flavouring Substances. Partial Agreement in the Social and Public Health Field. List N(1), Series I(b), no. 143, p. 18. Strasbourg.

Dabbah, R., Edwards, V. M. & Moats, W. A. (1970). Antimicrobial action of some citrus fruit oils on selected food-borne bacteria. *Appl. Microbiol.* **19**, 27.

Epstein, W. L. (1973). Report to RIFM, 30 October.

Flavoring Extract Manufacturers' Association (1965). Survey of flavoring ingredient usage levels. No. 2825. *Fd Technol., Champaign* **19** (2), part 2, 155.

Food Chemicals Codex (1972). 2nd ed. Prepared by the Committee on Specifications, Food Chemicals Codex, of the Committee on Food Protection. p. 565. National Academy of Sciences–National Research Council Publ. 1406. Washington, D.C.

Gildemeister, E. u. Hoffman, F. (1959). *Die Ätherischen Öle.* Vol. V, p. 530. Akademie Verlag, Berlin.

Guenther, E. (1949). *The Essential Oils.* Vol. III, p. 118. D. Van Nostrand, Inc., Princeton, New Jersey.

Homburger, F., Treger, A. & Boger, E. (1971). Inhibition of murine subcutaneous and intravenous benzo(rst)pentaphene carcinogenesis by sweet orange oils and d-limonene. *Oncology* **25**, 1.

Katz, A. (1946). *Spice Mill* **69** (July), 46.

Kesterson, J. W., Hendrickson, R. & Braddock, R. J. (1971). Florida Citrus Oils. Agricultural Experiment Stations. Bulletin 749 (technical), University of Florida, Gainesville.

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

Moreno, O. M. (1973). Report to RIFM, 4 September.

Pierce, W. E. H. (1959). The Role of Citrus Oils in Carcinogenesis. Thesis. University of London.

Roe, F. J. C. (1959). The effect of applying croton oil *before* a single application of 9,10-dimethyl-1,2-benzanthracene (DMBA). *Br. J. Cancer* **13**, 87.

Roe, F. J. C. & Field, Winifred E. H. (1965). Chronic toxicity of essential oils and certain other products of natural origin. *Fd Cosmet. Toxicol.* **3**, 311.

United States Pharmacopeia (1965). 17th revision. Prepared by the Committee of Revision. p. 427. The United States Pharmacopeial Convention, Inc., New York.

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

BITTER ORANGE OIL

Description and physical properties: EOA Spec. no. 155. The main constituent of bitter orange oil is *d*-limonene (Guenther, 1949).
Occurrence: Found in the fresh peel of the fruit *Citrus aurantium* Linne (Fam. Rutaceae).
Preparation: By the expression of the fresh peel of the fruit, *Citrus aurantium* Linne, by various methods, without the use of heat.
Uses: In public use since the 1900s. Use in fragrances in the USA amounts to less than 20,000 lb/yr.

Concentration in final product (%):

	Soap	*Detergent*	*Creams, lotions*	*Perfume*
Usual	0·02	—	0·02	0·25
Maximum	0·14	0·005	0·10	1·0

Analytical data: Gas chromatogram, RIFM nos 71-94 and 72-60; infra-red curve, RIFM no. 72-60.

Status

Bitter orange oil was granted GRAS status by FEMA (1965) and is approved by the FDA as GRAS for food use. The Council of Europe (1970) included bitter orange oil in the list of substances, spices and seasonings whose use is deemed admissible, with a possible limitation of the active principle in the final product. The *Food Chemicals Codex* (1972) has a monograph on bitter orange oil.

Biological data

Acute toxicity. The acute oral LD_{50} value in rats was reported as >5 g/kg (Owen, 1971a). The acute dermal LD_{50} value in rabbits was reported as >10 g/kg (Owen, 1971b).

Irritation. Undiluted bitter orange oil applied to the backs of hairless mice was very mildly irritating (Urbach & Forbes, 1972). Bitter orange oil applied full strength to intact or abraded rabbit skin for 24 hr under occlusion was moderately irritating (Owen, 1971b). Tested at 10% in petrolatum, it produced no irritation after a 48-hr closed-patch test in 25 human subjects (Kligman, 1971).

Sensitization. A maximization test (Kligman, 1966) was carried out on 25 volunteers. The material was tested at a concentration of 10% in petrolatum and produced no sensitization reactions (Kligman, 1971).

Phototoxicity. Distinct phototoxic effects have been reported for bitter orange oil (Urbach & Forbes, 1972).

Additional published data

Cutaneous irritation due to oil of bitter orange has been reported (Schwartz, Tulipan & Peck, 1947). A case of dermatitis has been reported in a girl employed to peel bitter orange; the condition was characterized by small vesicular eruptions on the fingers, hands, forearms and face (Murray, 1921). Sensitization of the skin of some individuals to light following the use of cologne waters containing oil of bitter orange has been reported (Szanto, 1928).

References

Council of Europe (1970). Natural and Artificial Flavouring Substances. Partial Agreement in the Social and Public Health Field. List N(1), Series 1(b), no. 136, p. 17. Strasbourg.

Flavoring Extract Manufacturers' Association (1965). Survey of flavoring ingredient usage levels. No. 2823. *Fd Technol., Champaign* **19** (2), part 2, 155.

Food Chemicals Codex (1972). 2nd ed. Prepared by the Committee on Specifications, Food Chemicals Codex, of the Committee on Food Protection. p. 566. National Academy of Sciences–National Research Council Publ. 1406, Washington, D.C.

Guenther, E. (1949). *The Essential Oils*. Vol. III. p. 197. D. Van Nostrand, Inc., Princeton, New Jersey.

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

Kligman, A. M. (1971). Report to RIFM, 17 June.

Murray, F. A. (1921). Dermatitis caused by bitter orange. *Br. med. J.* **1**, 739.

Owen, G. (1971a). Report to RIFM, 28 June.

Owen, G. (1971b). Report to RIFM, 23 August.

Schwartz, L., Tulipan, L. & Peck, S. M. (1947). *Occupational Diseases of the Skin*. Lea and Febiger, Philadelphia.

Szanto, J. (1928). Über Pigmentierunger nach Sensibilisierung der Haut mit ätherischen Ölen. *Zentbl. Haut- u. GeschlKrankh.* **28**, 451.

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

Fd Cosmet. Toxicol. Vol. 12, pp. 737-746. Pergamon Press 1974. Printed in Great Britain

Review Section

CHEMICAL CARCINOGENS IN THE ENVIRONMENT AND IN THE HUMAN DIET: CAN A THRESHOLD BE ESTABLISHED?*

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In the current atmosphere of concern over the possible deleterious effects of environmental chemicals to man, one encounters with increasing frequency the claim that, for certain compounds, there is no threshold level of exposure below which no harmful effects on an organism will occur. Such views can fairly easily be demonstrated to be toxicologically unsound under experimental conditions when acute and subacute toxicities are in question, since for these, clear-cut dose-response curves can be established for the dose levels tested in a variety of experimental animals. On the other hand, when argument centres around the induction of cancer by chemicals, the problem of defining a 'no-effect' exposure level becomes more complex and controversial.

In testing for long-term effects such as carcinogenicity, dose-response curves can, of course, again be constructed. Disagreements in this area of research begin over the meaning of the lower part of the curve and specifically over whether it is feasible to use laboratory studies with a limited number of test animals to determine a dose that would show no effect in a very large human population.

The nature of the problem as presented by critics is as follows. In carcinogenicity experiments, if one tests a particular substance at several dose levels (e.g. using 100 test animals for each dose), positive results can be expressed in a dose-response curve, with each point representing the percentage of animals that developed malignancies. However, even if one knows that at a given dose cancer was induced in 50% of the animals, at a lower dose in only 10% and at a still lower dose in none, the lowest dose cannot be regarded as a 'no-effect' threshold for induction of cancer in whole populations, since the results indicate only that none of the 100 test animals developed cancer at that dose rate. Further, if it has been shown that a substance can act as a carcinogen in test animals at some level, and if one assumes that animal experiments can be extrapolated to man, then one must seek a way of determining the lowest dose that would be safe not for 100 people, but for at least 100,000, or, in the more conservative view, for 100 million. Although there have been proposals to initiate experiments utilizing thousands or even millions of test animals,

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most researchers recognize such approaches as impracticable. As Weil (1972 & 1973) has pointed out, even supposing that one could establish the most probable shape or slope of a dose-response curve through the exposure of thousands of animals to low dose levels, such a slope would still involve uncertainties and would hold only for the specific experimental conditions tested. Furthermore, the determination of a low-risk, no-tumour-induction level for a chemical under a particular set of laboratory conditions would not obviate the need for agreement as to how one should extrapolate from findings with test animals to acceptable exposures for man. It is largely because of these problems with numbers, with the long induction time of effects, and with uncertainties regarding extrapolation, that some investigators engaged in cancer research have called the dose-response testing procedures of classical toxicology "crude and insensitive" when applied to the carcinogenicity testing of chemicals.

Three proposed methods for establishing a 'safe' exposure level

To circumvent the apparent inability to determine through experiment the possible effects on a very large population of regular exposure to exceedingly small quantities of a suspected carcinogen, it has been suggested that once a dose-response curve has been obtained for the carcinogenic potential of a chemical in the exposure range where effects are observable, the dose which should produce only one tumour in a population of 100,000, 1 million, or 100 million individuals could be calculated by downward mathematical extrapolation from the observable range. However, even such methods have been rejected by some as unreliable. The reasons for the doubtful validity of mathematically defined 'safe doses' are elaborated by the Technical Panel on Carcinogenesis in the *Report of the Secretary's Commission on Pesticides and their Relationship to Environmental Health*, hereafter referred to as the Mrak Report (Commission on Pesticides, 1969). The members of this panel cite material from the 1969 report of the Subcommittee on Carcinogenesis of the FDA Advisory Committee on Protocols for Safety Evaluation and announce that "the concerned statisticians at the National Cancer Institute concur" with the scepticism expressed in the FDA report. They quote (Mrak Report, p. 493): "It might be thought that the basis for such an extrapolation could be provided by observations in the observable range. To show how far from being the case this actually is, we give below three different dose-response curves, mathematically defined over a dosage range of 256-fold. All three have the same TD_{50} [dose which results in tumors in 50 percent of the animals] and TD_{16} [tumor dose for 16 percent of the animals]. The first is a probit curve, the second a logistic curve, and the third the so-called one-particle curve."

A recompilation of the two tables from the Mrak Report illustrating these three curves is presented in Table 1. Since the numbers listed under "Dose" are strictly abstract proportions, 1·0 stands for the dose which would produce tumours in 50% of the test animals, and all the other numbers represent multiples or fractions of that dose. The Panel (*ibid*, p. 494) comments: "It will be noted that below the TD_{50} the three curves differ by little and that in any experiment of practicable size (say less than several thousand animals) it would not be possible to conclude from the actual observations which one of the three best described the data. As shown below, however, the $TD_{.0001}$ (one in a million dose) and the $TD_{.000001}$ (one in one hundred million dose) obtained by extrapolation of these three curves differ markedly.... The one in one-hundred million dose, which Mantel and Bryan call the 'virtually' safe dose is one-hundredth the TD_1 using the probit curve, one-hundred thousandth using the logistic and one one-millionth using the one-particle curve."

Table 1. *Recompilation of tables from the report of the Technical Panel on Carcinogenesis**

Probit curve		Logistic curve		One-particle curve	
TD (%) on curve	Abstract dose	TD (%) on curve	Abstract dose	TD (%) on curve	Abstract dose
98·0	16·0	96·0	16·0	100·0	16·0
93·0	8·0	92·0	8·0	99·0+	8·0
84·0	4·0	84·0	4·0	94·0	4·0
69·0	2·0	70·0	2·0	75·0	2·0
50·0	1·0	50·0	1·0	50·0	1·0
31·0	0·5	30·0	0·5	29·0	0·5
16·0	0·25	16·0	0·25	16·0	0·25
7·0	0·125	8·0	0·125	8·0	0·125
2·0	0·062	4·0	0·062	4·0	0·062
1·0	0·04	1·0	0·022	1·0	0·0144
0·1	0·0155	0·1	0·00315	0·1	0·00144
0·001	0·00279	0·001	0·000063	0·001	0·0000144
0·0001	0·00136	0·0001	0·0000098	0·0001	0·00000144
0·000001	0·000412	0·000001	0·00000016	0·000001	0·000000014

*Mrak Report (Commission on Pesticides, 1969, p. 494).

The members of the Carcinogenicity Panel and the FDA statisticians further state that from data in the observable range, it is not possible to decide which of the three curves most accurately represents experimental findings, and that extrapolation downward to a "virtually safe dose" is therefore not reliable. The position of both publications is summarized in the following (*ibid*, p. 495): "Clearly extrapolation from the observable range to a safe dose has many of the perplexities and imponderables of extrapolation from animal to man and it would be imprudent to place excessive reliance on mathematical sleight of hand, particularly when the dose-response curves used are largely empirical descriptions, lacking any theoretical physical or chemical basis."

Variations on this kind of argument have been presented for the last 15 years or so by proponents of the 'no safe dose for carcinogens' school of thought. In 1970, for instance, in the report of the Ad Hoc Committee on the Evaluation of Low Levels of Environmental Chemical Carcinogens, the following claim is repeated several times (p. 1): "No level of exposure to a chemical carcinogen should be considered toxicologically insignificant for man. For carcinogenic agents a 'safe level for man' cannot be established by application of our present knowledge. The concept of 'socially acceptable risk' represents a more realistic notion."

And further (p. 4): "In order to evaluate the hazard of a chemical for man, one must extrapolate from the animal evidence. It is essential to recognize that no level of exposure to a carcinogenic substance, however low it may be, can be established to be a 'safe level' for man. This concept, put forward in the 1950's, remains true in 1970." Finally (p. 7): "It is impossible to establish any absolutely safe level of exposure to a carcinogen for man.... The principle of zero tolerance should be applied in all but the most extraordinary of cases."

On the face of things, the agnostic stance taken by the writers quoted would seem to rest on genuine 'unknowables', and the general conclusion that the only sure way to protect the public is through establishing zero tolerances would appear to express an attitude of prudence in the best tradition of preventive medicine. However, most of these arguments ignore the fact that in order to produce an effect, any material has to be present in a mini-

mum number of atoms, molecules, subatomic particles or photons (Dinman, 1972; Rossi & Kellerer, 1972). In the case of chemicals, the probability that one or even several hundred molecules of a compound will have a deleterious effect on an organism is virtually zero.

The threshold concept applied to human intake of DDT

In order to give concrete meaning to the figures resulting from the differing downward extrapolations presented in Table 1, one has to consider four parameters—a supposedly known carcinogen for which the TD_{50} has been established, the number of molecules present in the TD_{50} dose of the compound in question, the number of cells present in the organism for which the 'virtually safe dose' or the 'acceptable risk dose' needs to be established and the minimum number of molecules that must be present in a cell for their presence to be registered by that cell.

It has been claimed recently that DDT is a hepatocarcinogen, and, according to a report by Innes, Ulland, Valerio *et al.* (1969), the TD_{50} of DDT in mice is about 140 ppm in food. We propose to use DDT as an example for downward extrapolation, with 140 ppm in food as the first parameter, even though we entertain serious doubts whether it has actually been demonstrated to be carcinogenic.

The second parameter is easily established, since on the basis of Avogadro's Law, it is known that 1 g DDT contains $1{\cdot}7 \times 10^{21}$ molecules.

Next one should consider how many cells there are in the human body. Different types of cell vary considerably in size, but their weight can be calculated. Dinman (1972), for instance, states (after Hutchinson, 1964) that a typical liver cell weighs 7×10^{-9} g. From the data, however, one gathers that a spherical cell shape was assumed. Using a more realistic cylindrical form, one arrives at half of this value ($3{\cdot}5 \times 10^{-9}$ g) for the average liver cell. A red blood cell, which is of course much smaller, weighs only 9×10^{-11} g, and there are approximately 3×10^{13} red blood cells alone in the 6 litres of blood in the human body. The weight of an average body cell should be very close to 5×10^{-10} g, and if the weight of an average man is expressed as 7×10^{4} g and divided by the average cell weight, the result is $1{\cdot}4 \times 10^{14}$ total cells. Different approaches to estimating the number of cells in the human body lead to almost the same figure, so we take this to be a reasonable approximation.

The fourth question—that is, the number of molecules needed in order merely to be registered* by a cell—can be answered by considering the following. In 1964, Hutchinson compiled data regarding the numbers of different atoms in one liver cell. From his tabulation it became evident that atoms with a biologically demonstrable function are present in quantities of at least 10^{6}–10^{8}/cell. From these considerations he concluded: "... there might be too many commoner, accidentally and potentially interfering materials (or sites?) around in the cell for any very important substance to work practically at a concentration of less than 10^{4} atoms or molecules per cell" (Hutchinson, 1964).

A similar view was expressed by Dinman (1972), who showed, using both Hutchinson's approach and findings from enzyme-inhibition studies with potent inhibitors, that about 4×10^{4} molecules/cell are required to produce even a minimal degree of inhibition. He

*This term has nothing to do with an effect that can be measured, deleterious or otherwise. It is slightly awkward, but we wanted to avoid such anthropomorphic alternatives as "noticed by the cell", whuch conveys the meaning with more linguistic smoothness but has the disadvantage of implying that some kind of conscious recognition is involved.

wrote: "To believe that a single molecule's presence in a cell implies a definite potential for deleterious effect disregards stochastic considerations.... While the construction of stochastically sound model is remote, the reasonableness of the hierarchy of cellular element concentrations as these relate to metabolic function suggest that a threshold for biological activity exists within a cell at 10^4 atoms.... The cellular organism operates within a quantitative rate limit that transcends any statements having only qualitative bases. Thus concepts concerning encroachment on response capabilities over a life span are inadequate descriptors of biological activity in the absence of quantitative qualifiers."

Some reservations might be raised about these generalizations if consideration is given to certain compounds which are in constant circulation, are target specific and undergo little metabolic transformation or excretion. Uptake of minute quantities of some of these oligodynamic substances is adequate to maintain homeostasis of an organism. In order to examine whether or not a cellular threshold exists at about 10^4 molecules for such chemicals, we selected vitamin B_{12} as an example, utilizing data from Kuschinsky & Lüllmann (1972). For the purpose of illustration we shall relate molecular quantities of the vitamin to numbers of mature erythrocytes, although it is recognized that vitamin B_{12} primarily affects the haemopoietic system. The underlying assumptions and values for all the calculations in the text are summarized in Table 2.

Table 2. *Summary of values and assumptions used in text*

Parameter	Value
Avogadro's no. (molecules)	$6{\cdot}02 \times 10^{23}$
Mol wt	
DDT	354·5
Vitamin B_{12}	1355·4
DAB	225·3
MC	268·3
AB	197·2
Weight (g)	
Liver cell	$3{\cdot}5 \times 10^{-9}$
Erythrocyte	9×10^{-11}
Average body cell	5×10^{-10}
Man*	
Body weight (g)	7×10^{4}
No. of cells in:	
Liver	5×10^{1}
Blood	3×10^{3}
Body	$1{\cdot}4 \times 10^{14}$
Rat*	
Body weight (g)	$2{\cdot}0 \times 10^{2}$
No. of cells in:	
Liver	$1{\cdot}4 \times 10^{9}$
Body	$4{\cdot}0 \times 10^{11}$
Mouse*	
Body weight (g)	$5{\cdot}0 \times 10^{1}$
No. of cells in:	
Liver	$3{\cdot}5 \times 10^{8}$
Body	$1{\cdot}0 \times 10^{11}$

DAB = Dimethylaminoazobenzene
MC = 3-Methylcholanthrene
AB = 4-Aminoazobenzene

*The assumptions on weights and cell numbers are approximations which are clearly subject to wide variation.

The average daily intake of vitamin B_{12} in man is about 1 μg, which contains $4{\cdot}5 \times 10^{14}$ molecules, or only 3 molecules/body cell. However, this quantity is added to an already existent vitamin pool in the body, comprising about 2·5 mg (c. 6×10^{17} molecules), half of it in the circulation. The small amount of vitamin B_{12} in the daily diet can be taken up by man because it immediately complexes with intrinsic factor at the site of uptake in the ileum. On the other hand, should the quantity of vitamin B_{12} in the blood fall below 10% of the normal value ($<6 \times 10^{16}$ molecules in the total circulation or 2×10^3 molecules/red corpuscle), as is the case in pernicious anaemia, the therapeutic doses recommended to restore normal circulating levels are from 30 to 100 μg/day, given intramuscularly. This amounts to between 500 and 2000 molecules/erythrocyte, and since excretion of the vitamin is minimal ($<0{\cdot}3$ μg/day), within a few days such administration should bring the blood level above 10^4 molecules/erythrocyte. One aspect of these figures is noteworthy, in that in the normal blood there are only 2×10^4 molecules of the vitamin for each erythrocyte—a figure very close to that given by Dinman (1972) for the concentration at which inhibitory molecules can produce minimal enzyme inhibition. Furthermore, if the number of vitamin B_{12} molecules/red blood cell falls by less than one order of magnitude (2×10^3), pernicious anaemia generally develops.

From the above considerations, about 4×10^4 molecules/cell seems to be a reasonable figure for the fourth parameter. We may now proceed to a consideration of the absolute quantities of DDT involved in the two safest exposure levels derived from the three different curves described in the Mrak Report (Table 3).

If the figures for the three curves in Table 1 are utilized and the 1 in 1 million ($TD_{0{\cdot}0001}$) as well as the 1 in 100 million ($TD_{0{\cdot}000001}$) induction doses for tumours caused by DDT are considered according to the tabulated values for downward extrapolation, it can be seen from Table 3 that none of the three curves yields a dose which, dispersed in the body, reaches the concentration of 4×10^4 molecules/body cell. Even were we to assume that the approximate 1·5 kg daily food intake of a man would be uniformly contaminated with DDT and, in addition, that the whole 'dose' of the compound would enter the liver directly in unchanged form (both of which assumptions are obviously untenable), only extrapolations from the probit curve would result in values above those needed merely to be registered by the liver cells.

Table 3. *Extrapolations to "safe doses" of DDT with a TD_{50} of 140 ppm in the diet*

Tumour incidence on	Dose in food (ppm)	Quantity of DDT in 1·5 kg food: Total dose, μg	Total dose, Molecules	No. of molecules: Per human body cell	No. of molecules: Per human liver cell
Probit curve					
$TD_{0{\cdot}0001}$	0·18	270	$4{\cdot}6 \times 10^{17}$	$3{\cdot}3 \times 10^3$	$9{\cdot}2 \times 10^5$
$TD_{0{\cdot}000001}$	0·056	84	$1{\cdot}4 \times 10^{17}$	$1{\cdot}0 \times 10^3$	$2{\cdot}8 \times 10^5$
Logistic curve					
$TD_{0{\cdot}0001}$	0·0014	2	$3{\cdot}4 \times 10^{15}$	24	$6{\cdot}8 \times 10^3$
$TD_{0{\cdot}000001}$	0·000023	0·034	$5{\cdot}9 \times 10^{13}$	$4{\cdot}2 \times 10^{-1}$*	$1{\cdot}2 \times 10^2$
One-particle curve					
$TD_{0{\cdot}0001}$	0·0002	0·3	$5{\cdot}1 \times 10^{14}$	3·6	$1{\cdot}0 \times 10^3$
$TD_{0{\cdot}000001}$	0·000002	0·003	$5{\cdot}1 \times 10^{12}$	$3{\cdot}6 \times 10^{-2}$†	10

*Or approximately 1 molecule/3 cells.
†Or approximately 1 molecule/27 cells.

The threshold of certain known chemical carcinogens

Considerations related to those presented above were reported by the Food Protection Committee (1960), both in connexion with the feeding studies carried out by Druckrey (1959) on rats, using dimethylaminoazobenzene (DAB; butter yellow) and with results from experiments on mice (Bryan & Shimkin, 1943) using single injected doses of 3-methylcholanthrene (MC), dibenz[*a,h*]anthracene (DBA), and benz[*a*]pyrene (BP). Druckrey (1959) found that lifetime feeding of 0·3 mg DAB daily to rats had no hepatic tumorigenic effect, whereas 1 mg of the dye fed daily over the lifespan of the animals resulted in a 20% incidence of hepatic tumours. In the single injection studies with mice (Bryan & Shimkin, 1943), the 'no-effect levels' for the production of sarcomas for the three compounds studied were established as 4 μg for MC, 2 μg for DBA and 30 μg for BP.

The Food Protection Committee (1960) pointed out that in Druckrey's studies a lifetime intake (summation of the daily dose for 700 days) of 10^{12} molecules DAB/rat-liver cell was necessary to induce a 20% incidence of hepatomas in the animals, whereas 10^{11} molecules/cell was ineffective. To be more precise, however, it should be mentioned that the same total number of molecules produced some tumours when higher doses were administered for a shorter time. Concerning the findings of Bryan & Shimkin (1943) with BP, the Committee wrote (p. 14): "...the injection of approximately 6×10^{16} molecules did not produce sarcomas, whereas the injection of $1{\cdot}2 \times 10^{17}$ molecules did". The Committee's comment on the difference between the molecular quantities of injected BP that had no tumorigenic effect and the quantities that did induce sarcomas is an important point.

Taking a slightly different approach to the DAB findings of Druckrey (1959), the individual daily dose which produced no effects may be considered; this dose is 0·3 mg/rat and it represents $5{\cdot}7 \times 10^8$ molecules/liver cell. The level at which 20% of Druckrey's animals developed hepatic tumours was 1 mg/rat/day—a presumed exposure of $1{\cdot}9 \times 10^9$ molecules/liver cell. According to the Food Protection Committee (1960), one may assume that only 1% of the DAB reaches the liver unchanged, which would imply that the no-effect level is $5{\cdot}7 \times 10^6$ molecules/rat-liver cell, whereas a daily exposure of $1{\cdot}9 \times 10^7$ molecules/liver cell over the lifespan of the rat is necessary for induction of a 20% incidence of hepatoma.

These figures on the minimal exposure to ingested DAB leading to liver tumours seem to be in excellent agreement with the data emerging from the extensive studies of Terayama (1967) on DAB and its analogues. Following a 50 mg single intragastric administration to rats, Terayama found that with DAB itself, which has been described as "highly carcinogenic", 28 μg of the compound was bound to protein in a 7 g rat liver at the time of peak recovery. This indicates that $5{\cdot}4 \times 10^7$ molecules/liver cell were present. At the same dose level, the "weakly carcinogenic" analogue of DAB—2′-methyl-4-monomethylaminobenzene (me-MAB)—gave a peak protein-bound dye recovery of only 11 μg/rat liver, while its carcinogenic activity was one-sixth of that of DAB. A low tumour incidence thus resulted from 2×10^7 molecules of the compound/liver cell (cf. the Druckrey (1959) study, in which $1{\cdot}9 \times 10^7$ molecules/liver cell in the feeding experiments also led to a relatively low (20%) incidence of hepatoma). Finally, when the non-carcinogenic DAB analogue, 4-aminoazobenzene (AB), was tested at the same dosage, the recovery of protein-bound compound in the rat liver was less than 2 μg. This quantity corresponds to $4{\cdot}4 \times 10^6$ molecules of the chemical for each rat-liver cell and compares with the no-effect DAB dose of $5{\cdot}7 \times 10^6$ molecules calculated earlier from the figures of Druckrey (1959).

DAB and its analogues are the most thoroughly studied and probably the best understood of all known or suspected chemical carcinogens. The binding of the dye molecules to a highly specific protein in liver-cell sap has been established. Speculations have been advanced that the dye-binding protein is a repressor or has the nature of an apo-repressor. In further experiments with DAB, Terayama (1967) found that as he lowered the starting dose, protein-bound recovery was correspondingly decreased, and the chemical ceased to act as a carcinogen. The lowest protein-bound recovery level for all three compounds mentioned in the foregoing (DAB, me-MAB, AB) was about 1 μg/rat liver.

Other statistical approaches

Returning to the subject of proposals for the statistical analysis of experimental data, on the basis of which downward extrapolations may be carried out to establish 'safe' levels of human exposure to carcinogenic agents, some other methods should be discussed. In 1961, Mantel & Bryan utilized the findings of Bryan & Shimkin (1943) on MC to calculate a "virtually safe dose" (VSD) for MC. They defined the VSD as an exposure level that would give rise to only one tumour in a population of 100 million individuals. They employed a probit curve with 99% confidence limits, arriving at a VSD for MC of 9×10^{-8} mg (or 90 pg)/mouse. This same quantity was considered to be the VSD for man (Mantel & Bryan, 1961). MC, unlike DAB, is not liver-specific in its effects. If this dose were dispersed in the whole human body, the exposure would amount to 1 molecule for every 700 body cells. Should the 90 pg of the compound enter the liver in its totality, the VSD would still yield only 2 molecules for every five human liver cells.

In 1970, Schneiderman presented a somewhat different proposal for estimating an "acceptable risk dose" (ARD), which was incorporated into the report of the Ad Hoc Committee (1970) as Appendix 3. His procedure is based on use of the one-particle curve with an upper 95% confidence limit, and the lower section of the curve is constructed to pass through the origin (no dose, no response). From results obtained in animal experiments, he extrapolates only to the dose which should produce one tumour in 100,000 individuals ($TD_{0.001}$). On the other hand, when extrapolating from animal to man, he suggests: "...that an appropriate 'safety factor' for man should involve a reduction in the ARD for animals by a factor of 100 to allow for species' differences, another factor of 100 to allow for interactions with other carcinogens, and another factor of 100 to hedge against the incorrect choice of 'blow up' (weight or surface area) from animals to man. This would imply an ARD in man of about 1×10^{-6} the ARD in animals" (Schneiderman, 1970).

Utilizing these assumptions, the ARD of DDT for man would be 3 pg total body dose, or $5{\cdot}1 \times 10^{9}$ molecules. This exposure, if dispersed throughout the body, would give 1 molecule for every 27,500 cells, or, if concentrated in the liver, 1 molecule for every 100 cells. It need hardly be emphasized that human exposure to such a quantity of this supposed carcinogenic pesticide does not appear to have any significance, especially in the light of Dinman's work (Dinman, 1972) on the threshold of effect for individual cells.

However, if one takes a converse approach and examines the exposures to DDT which would be 'unsafe', accepting the 10^{-6} safety factor for man suggested by Schneiderman (1970) and applying his preferred curve, a serious problem emerges. According to his method, the exposure which should cause tumours in 1 in 100 individuals (TD_1) is a total dose of 30 ng/man/day. In view of the fact that the whole human population of the earth has been ingesting at least 1000 times this quantity of the pesticide daily for the past 25 years, one would expect that were DDT indeed carcinogenic for man, and were these safety

assumptions based in reality, there ought to have been a massive increase in primary liver cancer among humans in the age group at greatest risk, that is, in those who are now between 50 and 70 years old. In effect, the average daily intake of DDT by man over these 25 years would correspond approximately to Schneiderman's TD_{50}, and thus, in the population at risk, exposed for at least a quarter of their lives, every eighth person should have developed hepatocarcinoma.

It is generally accepted that MC is a relatively potent carcinogen. When the socially acceptable risk is calculated according to Schneiderman (1970), the ARD value for MC exposure is $3{\cdot}15 \times 10^{-6}$ pg. While the author admits that his procedure "...gives a somewhat more conservative answer than the procedure of Mantel and Bryan" (Schneiderman. 1970), this concession must be regarded as an understatement, considering that the VSD for MC derived by Mantel & Bryan (1961) is 90 pg, a quantity which is 3×10^{7} times greater than the ARD derived by utilizing Schneiderman's approach. The ARD value of $3{\cdot}15 \times 10^{-6}$ pg for MC, when expressed in terms of molecules, yields 1 molecule for every 20 billion cells in the human body. Mathematical methods yielding such conclusions do not seem to have any relevance to human biology.

The newest version of these mathematical techniques was proposed by the FDA and published in the Federal Register of 13 July 1973. Basically, the method involves a linear extrapolation downward from data in the observable range to a 1 in 100 million risk dose. However, for the lower ranges a more conservative slope is used, which results from application of the lower 90% confidence limit in constructing the curve. Starting once again from the findings of Bryan & Shimkin (1943) on MC, the socially acceptable dose would be 120 ng, or 2 molecules of the compound for each body cell, if the probit line were to be extrapolated in a linear fashion to the $TD_{0{\cdot}000001}$. However, since the slope of the lower portion of the curve is skewed to 'make allowance for the sampling error', the acceptable exposure is reduced to 3·8 ng, or 1 molecule for every 16 body cells.

Concluding remarks

From the data discussed earlier, it seems probable that a hepatocellular threshold level for tumour induction exists at approximately 2×10^{6} molecules/liver cell, irrespective of the route of administration, at least for DAB and its analogues. If equally detailed information becomes available on other substances which have been demonstrated in well-designed animal experiments to be strongly carcinogenic, it may become feasible to establish a threshold exposure level for the carcinogenic transformation of cells.

We voice our agreement with the statement cited earlier from the Mrak Report (although for quite different reasons from those of the original writers) that in assessing safe levels of human exposure to chemical carcinogens "...it would be imprudent to place excessive reliance on mathematical sleight of hand" (Mrak Report, p. 495). Setting exposure standards on the basis of statistical extrapolations which fail to take account of fundamental biological concepts, such as the principle of threshold, can be highly misleading, resulting in unnecessarily conservative 'permissible exposures', while at the same time failing to resolve the issue of determining the quantitative level at which chemical carcinogens will not have a deleterious effect on a living organism.

REFERENCES

Ad Hoc Committee on the Evaluation of Low Levels of Environmental Chemical Carcinogens (1970). Report to the Surgeon General. US Department of Health, Education, and Welfare, Washington, D.C.

Bryan, W. R. & Shimkin, M. B. (1943). Quantitative analysis of dose-response data obtained with three carcinogenic hydrocarbons in strain C3H male mice. *J. natn. Cancer Inst.* **3**, 503.

Commission on Pesticides (1969). *Report of the Secretary's Commission on Pesticides and Their Relationship to Environmental Health.* Parts I and II. US Department of Health, Education, and Welfare. Washington, D.C.

Dinman, B. D. (1972). "Non-concept" of "no-threshold": Chemicals in the environment. Stochastic determinants impose a lower limit on the dose-response relationship between cells and chemicals. *Science, N.Y.* **175**, 495.

Druckrey, H. (1959). Pharmacological approach to carcinogenesis. In *Ciba Foundation Symposium on Carcinogenesis: Mechanisms of Action.* Edited by G. E. W. Wolstenholme & Maeve O'Connor. p. 110. Little, Brown & Co., Boston.

Food Protection Committee (1960). Problems in the evaluation of carcinogenic hazard from use of food additives. NAS–NRC Publn 749. Washington, D.C.

Hutchinson, G. E. (1964). The influence of the environment. *Proc. natn. Acad. Sci. U.S.A.* **51**, 930.

Innes, J. R. M., Ulland, B. M., Valerio, Marion, Petrucelli, L., Fishbein, L., Hart, E. R., Pallotta, A. J., Bates, R. R., Falk, H. L., Gart, J. J., Klein, M., Mitchell, I. & Peters, J. (1969). Bioassay of pesticides and industrial chemicals for tumorigenicity in mice: A preliminary note. *J. natn. Cancer Inst.* **42**, 1101.

Kuschinsky, G. & Lüllmann, H. (1972). *Pharmakologie.* 5th ed. Georg Thieme Verlag, Stuttgart.

Mantel, N. & Bryan, W. R. (1961). "Safety" testing of carcinogenic agents. *J. natn. Cancer Inst.* **27**, 455.

Rossi, H. H. & Kellerer, A. M. (1972). Radiation carcinogenesis in low doses. *Science, N.Y.* **175**, 200.

Schneiderman, M. A. (1970). A method for determining the dose compatible with some "acceptable" level of risk. In: Report to the Surgeon General by the Ad Hoc Committee on the Evaluation of Low Levels of Environmental Chemical Carcinogens. p. 11. US Department of Health, Education, and Welfare, Washington, D.C.

Terayama, H. (1967). Aminoazo carcinogenesis—Methods and biochemical problems. In *Methods in Cancer Research.* Vol. I. Edited by H. Busch. p. 399. Academic Press, New York.

Weil, C. S. (1972). Statistics vs safety factors and scientific judgment in the evaluation of safety for man. *Toxic. appl. Pharmac.* **21**, 454.

Weil, C. S. (1973). Experimental design and interpretation of data from prolonged toxicity studies. In *Pharmacology and the Future of Man.* Proc. 5th Int. Cong. Pharmacol. San Francisco, 1972, Vol. 2, p. 4. S. Karger, Basel.

Fd Cosmet. Toxicol. Vol. 12, pp. 747–748. Pergamon Press 1974. Printed in Great Britain

REVIEWS OF RECENT PUBLICATIONS

Report of the Government Chemist 1972. Department of Trade and Industry: Laboratory of the Government Chemist. HMSO, London, 1973. pp. vi + 180. £1.15.

The work of the Laboratory of the Government Chemist covers a wide field, embracing agricultural materials, food and nutrition, waters and waste waters, drugs and medicines, bacteriology, dental materials, smoking, activities in connexion with Customs and Excise, radiochemistry and a variety of general chemical services and physical methods of analysis.

In the period reported, about 40% of the work was devoted to environmental matters, with pesticides remaining a major preoccupation. During the year beginning October 1970, the laboratory was engaged in a second total-diet study of pesticide residues (the first having been conducted in 1966–67). This second study revealed total levels of 27 μg organochlorine pesticides and 53 μg malathion—the only organophosphorus pesticide identified—in the average daily diet. Of the organochlorines, *p,p'*-DDT was present overall at the highest mean level (0·0045 ppm), followed by *p,p'*-DDE and γ-BHC, although different patterns were evident in specific foodstuffs. The results showed that levels of dieldrin and of DDT and its degradation products had decreased since the 1966–67 study, whereas γ-BHC had remained approximately constant and malathion had increased markedly, a change probably reflecting a great increase in the use of Australian grain. In all cases, however, levels were well below the acceptable daily intakes established by FAO/WHO.

Polychlorinated biphenyl (PCB) residues were also discovered during the total-diet study, but were present in only 13/385 samples and at levels of only 0·1 ppm or below. Fish alone showed their consistent presence, at levels in the range 0·01–0·08 ppm. In all foodstuffs, including meat fat and cod-liver oil analysed outside the total-diet study, residues were well within the guideline levels established in the USA by the FDA. Somewhat surprisingly, no PCB could be detected in 25 samples of packaging materials, down to the limit of sensitivity of 1 ppm, perhaps because of its absence from current UK types of carbonless copy paper (the main source of such contamination in the USA). Human fat, too, contained less than 1 ppm PCB, again in contrast to the situation in the States.

The Food and Nutrition Division of the Laboratory has been working for some years on a revision of *The Composition of Foods* by R. A. McCance and E. M. Widdowson (HMSO, London, 1960). Analysis of meat and meat products is now well advanced and work on fish is about to begin, to be followed by an examination of miscellaneous foodstuffs. It is anticipated that the analytical work will be completed during 1974, in readiness for publication of the new edition of the tables in 1975. The Division has also been deeply involved in analysing foodstuffs for lead and cadmium on behalf of the Working Party on The Monitoring of Foodstuffs for Heavy Metals, reports by which have now been published (*Cited in F.C.T.* 1973, **11,** 123; *ibid* 1974, **12,** 139). Lead and cadmium have also figured in the work of the General Chemical Services Division, which has analysed coloured gummed paper for use in schools, and the glaze on table and oven ware. As a result of this work, coloured gummed paper will be added to the list of materials which should contain no more than 250 ppm lead or other toxic metals, and regulations will be introduced to limit extractable lead and cadmium from glazed culinary ware.

In the veterinary field, the Laboratory has been working on methods for the determination of feed-additive residues, and a method for carbarsone is outlined in the Report. EEC official methods for the analysis of fertilizers and feeding stuffs have also been examined, and modifications or alternatives have been suggested where necessary. Special attention has been given to biological methods for determining antibiotic residues in poultry, and to the substantiation of assay methods for prophylactic levels of antibiotics in feedstuffs. In the case of pesticides, a method has been developed for determining 2,3,7,8-tetrachlorodibenzo-*p*-dioxin in 2,4,5-T and its esters and in finished formulations, and levels below 1 ppm have been found in all but one instance. It is now hoped to extend the work to other chlorinated dibenzodioxins, and to examine other chlorophenol-derived pesticides.

Other work at the Laboratory has involved methods for determining the biodegradation of nonionic detergents, and the determination of fluoride in potable waters using an ion-selective electrode. The development of field tests for the determination of toxic substances in factory atmospheres has continued, particular attention being paid to the aromatic amines, 1-naphthylamine, di-(4-amino-3-chlorophenyl)methane (MOCA) and *N,N*-dimethylaniline, and to lead, the threshold limit value for which has recently been lowered from 0·2 to 0·15 mg/m^3. Heavy metals, particularly arsenic and cadmium, in factory fume emissions have also been investigated on behalf of the Alkali and Clean Air Inspectorate.

The diversity of subjects studied by the Laboratory, and the importance of the work undertaken, should render this report of interest to a very wide audience.

Fd Cosmet. Toxicol. Vol. 12, pp. 749–756. Pergamon Press 1974. Printed in Great Britain

BOOK REVIEWS

Sorbinsäure. Chemie—Biochemie—Mikrobiologie—Technologie—Recht. Band 4. Lebensmittelrechtliche Zulassungen. By E. Lück. B. Behr's Verlag, Hamburg, 1973. pp. 99.

Contrary to our statement in an earlier issue (*cited in F.C.T.* 1973, **11,** 887), this project on sorbic acid was not completed when the third volume in the series appeared. Volume 4, concerned with legislation relating to the use of sorbic acid as a food preservative, was in fact in preparation, and towards the end of 1973 it joined the already published volumes on the chemistry, on the biochemistry, physiological effects and microbiology and on the uses and technology of this important preservative.

As with many other food additives, the food law provisions for sorbic acid and its salts and derivatives vary widely from country to country, both as regards the types of food in which they may be used and the maximum levels permitted. The aim of the current exercise was to cover the provisions of all states for which reliable information existed, and the result is a series of summaries of sorbic acid legislation for 58 countries or other legislative areas, including the EEC and the individual Australian states. For each authority, a brief introductory paragraph is followed by a table listing the foods allowed to contain sorbic acid and the maximum permitted levels in each case. The piece of legislation relevant to each statement is identified and footnotes fill in certain other essential details.

Anyone who has tried to summarize legislation in this way will be aware of some inherent difficulties. To begin with, the complexity of much national legislation is not readily reduced to a simple tabulation. The author's introductory remarks make it clear that he has largely avoided this problem—and restricted the size of the book—by omitting not only provisions on the combined use of sorbic acid with other substances but also various other special declarations and limitations, such as those relating to particular provisions for the use of the preservative in specially packed foods.

Another pitfall is the difficulty of ensuring the authenticity of material translated from a wide variety of languages. In particular, many types of food of major importance in some areas may have no exact counterpart in other languages. Wherever possible, therefore, the regulations and other reference sources have been studied in the original and, while the basic text of this publication is in German, the names of foods are listed in the tables also in the original language for all countries using the Latin script. For other countries the list is given in English as well as German, whenever an official English translation of the legislation exists, while for the rest the foods are listed only in German, using as literal a translation as possible.

The third major problem in a task of this nature is the rapidity with which legislation tends to change. There is little an author can do about this beyond recognizing, as Dr. Lück does, that there may be some inaccuracies and omissions in his text and stating clearly the time limits on the information provided. The material reproduced in this volume reflects the situation in the autumn of 1972.

Dr. Lück's handling of this whole project has demonstrated that he is undaunted by tasks involving the detailed and meticulous handling of extensive collections of data, and

he has tackled this latest exercise systematically and with a clear recognition of the difficulties. It would perhaps not be over optimistic, therefore, to speculate that if in the future he finds this survey of legislation becoming seriously outdated, he may be prepared to undertake the revision of what many are likely to find a very useful little book.

Plastics in Contact with Food. By J. H. Briston and L. L. Katan. Food Trade Press Ltd., London, 1974. pp. xii + 466. £12.

The authors have chosen as the theme of their book "How can plastics assist in delivering food from its source to the consumer economically, safely and of unimpaired quality?". With the present world shortage of food inevitably growing more acute, the importance of this topic can be clearly recognized. The protection of food against loss caused by natural agents and the prevention of bacterial and chemical contamination of food are major benefits conferred by the use of plastics.

This publication is intended primarily as a textbook for technologists. Thus the bulk of the publication is composed of chapters relating to raw materials—what they are and how they are made—and the ways in which they can be utilized to provide articles and systems that will help to improve food distribution. Clearly there are many very different problems to be resolved before any plastics material is finally brought into use. The book aims to give practical help on these problems and to dispel some of the common misconceptions about hazards by focusing attention on the positive side of the picture.

Hazards of course include the possibility that foodstuffs may be contaminated by chemicals migrating from the plastics and may therefore carry potential problems of taint and toxicity. The need in this connexion for expertise outside the field of plastics technology is clearly recognized by the authors. Some basic information has been included to enable the reader to appreciate the toxicological problems involved, but wisely no attempt has been made to tackle this aspect in any detail. The systematic approach to the problem of migration should, however, prove of considerable interest to those involved in this field. Of interest also are the different ways in which various countries have tackled the problems of determining the safety of plastics intended for food-contact use and of harmonizing the scientific and safety aspects of food-packaging with existing legislative systems, local customs and social habits. The measures taken are illustrated by a comprehensive review of the position and general approach in about thirty countries.

Although the authors have addressed themselves primarily to the technologist, many readers remote from the industrial laboratory should find much of interest in this volume.

Tissue Culture. Methods and Applications. Edited by P. F. Kruse, Jr. and M. K. Patterson, Jr. Academic Press, New York, 1973. pp. xxvii + 868. £10.15.

Ever since 1907, when R. G. Harrison succeeded in growing frog embryonic nerve fibres, the science of tissue culture can truly claim to have been a growth industry. Recently the development of new techniques has been explosive, and today tissue and cell cultures provide valuable experimental tools for a variety of biological disciplines from virology and immunology to toxicology and cancer research. The aims of this book were to collate some of the newer methods of tissue culture and include representative protocols for the application of these techniques. The editors have achieved this objective by producing a treatise

to which more than 100 authors have contributed, and by selecting the more up-to-date procedures for consideration, some 80% of those included having been derived from research carried out during the last 8 years.

The book is divided into 14 sections, the initial ones covering general topics such as dissociation and the harvesting, cloning and replication of cells. Subsequent sections deal with system automation, mass culturing, evaluation of culture dynamics and recent techniques for light and electron microscopic observation of cells Then follow protocols for the use of tissue culture techniques in specific disciplines. These include cell hybridization, virus propagation, hormone production and the diagnosis of disease. The final section deals with quality control measures, such as sterility testing, control of sera, and detection of pathogens in cultures and their elimination. In total, over 150 separate protocols are described.

The editorial comments introducing each section are useful and supply a certain amount of cross-referencing. For such a large and diverse volume, however, these general comments are all too brief. On the whole, the protocols described are clearly written and should prove relatively easy to follow, providing the appropriate and often complex equipment is available. Excellent illustrations, both photographic and diagrammatic, are an attractive and invaluable feature of many of the contributions. It is also refreshing to find that many authors emphasize the potential and sometimes unavoidable sources of error in their protocols, thereby providing a critical and objective approach to the techniques described.

It is obviously impossible in one volume to include all the current applications of tissue culture, but one exciting and rapidly growing field mentioned only briefly is the development of *in vitro* techniques designed to detect potential mutagens and carcinogens. More emphasis on these investigations would have enhanced the value of this compendium. Nevertheless, the book can be strongly recommended, as an important work of reference, both to experienced users of culturing techniques and to those working in the field for the first time.

The Macrophage. A Review of Ultrastructure and Function. By I. Carr. Academic Press, London, 1973. pp. viii + 154. £5.20.

The macrophage attracted the early attention of microscopists because of its resemblance to the free-living amoeba. Not only could 'amoeboid' movements be detected in both types of cell but ingestion of neutral particulate material could be readily induced in both.

Particular attention has been paid in the first and last chapters of the book named above to defining the macrophage in the light of current knowledge and to distinguishing between macrophages and macrophage-like cells. This distinction is particularly useful in view of the considerable confusion that has arisen in the past between these two types of cell. Another useful feature is the description of 'fixed' macrophages in various organs. The literature on this topic is widely scattered through a variety of publications from clinical journals to highly specialized ones on tissue culture. The author has managed to bring together the more important of these data and to place in perspective information that might otherwise appear irrelevant or even contradictory.

The controversial topic of the origin of the macrophage is succinctly treated in a review covering the origin of both the free and the 'fixed' macrophage. A greater attempt could

perhaps have been made here to provide a critical assessment of the thinking behind the experiments mentioned, but one has to accept that this is a difficult field and such an approach would not have been wholly in keeping with the rest of the book, which is essentially of a practical rather than a philosophical nature.

The well-established functions of the macrophage, namely phagocytosis and pinocytosis, are clearly outlined and the dominant role played by the cell in some chronic inflammatory lesions, both naturally occurring and experimentally induced, is well brought out. More recently discovered functions of the macrophage—the secretion of antibacterial substances and participation in iron and lipid metabolism—are outlined in two brief chapters, and of interest particularly to the pathologist is a review of evidence suggesting that two types of tumour, reticulum-cell sarcoma and Hodgkin's disease, originate in the macrophages.

On the whole this book may be recommended to biologists and pathologists who wish to look further, but not too deeply, into the current knowledge on these important cells, which are prominent in any reactive lesion caused in the body either by external agents or by some internal aberration. The author's clear and concise style will help the reader round many a difficult corner.

The Excretory Function of Bile: The Elimination of Drugs and Toxic Substances in Bile. By R. L. Smith. Chapman and Hall Ltd., London. pp. xii + 283. £5.

As the first extensive review that has appeared on the very important subject of the biliary excretion of xenobiotics, this book is very welcome. The author is an acknowledged expert in the field, having contributed extensively to the literature on this topic during the past 10 years in collaboration with Professor R. T. Williams, who has written the Foreword to the book. In the past, the biliary route of excretion of foreign compounds has not received a great deal of attention from toxicologists, but it is now becoming increasingly recognized as an important factor in the overall elimination of drugs, pesticides and food additives.

The first part of the book deals with the principles of biliary excretion, starting with the anatomy and physiology of the liver and the processes involved in bile production. This leads on to a consideration of the factors affecting the excretion of substances in the bile, including the physico-chemical characteristics of the compounds concerned, the mechanisms involved and the importance of various metabolic reactions in the transfer of substances from liver to bile, and the influence of protein binding and urinary excretion on biliary excretion. An interesting chapter on inter-species variations in biliary excretion is included in this part of the book. It was this aspect of the subject that first focused attention in the Department of Biochemistry, St. Mary's Hospital Medical School, on the problems and complexities of biliary excretion, and thus led indirectly to this volume. All the major points discussed in this section are comprehensively illustrated by reference to a wide range of compounds.

In the second section of the book, the author has attempted to bring together information on the biliary excretion of specific foreign compounds published in the literature up to and including 1971. The substances covered range from simple aliphatic compounds, such as dimethyl sulphoxide and chlorinated hydrocarbons, to drugs (including sulphonamides, antibiotics and cardiac glycosides), pesticides, azo dyes (long used in the food, cosmetic and pharmaceutical industries) and polycyclic hydrocarbons. The style of pre-

sentation is consistent throughout: each compound's structural formula is reproduced and its uses are outlined before the known data on biliary excretion are briefly reported. Any unusual or noteworthy points are stressed and a comprehensive bibliography concludes each chapter. The complete volume has a subject and author index.

This book should prove valuable to students and research workers in the fields of toxicology, pharmacology, biochemistry and pharmacy, and to others concerned with the effects and safety of food additives, pesticides and drugs. Its content, high standard of presentation and reasonable price should make it a classic in its field.

Isolated Liver Perfusion and its Applications. Edited by I. Bartošek, A. Guaitani and L. L. Miller. Raven Press, New York; distributed by North-Holland Publishing Co., Amsterdam, 1973. pp. 283. $21.20.

This textbook on liver perfusion should be comprehensible to anyone with a knowledge of the biological sciences. The editors are supported by 82 other authors, mainly from Europe but in a few cases from the USA and Canada, who contribute papers dealing with different aspects of liver perfusion.

Early papers give a comprehensive description of the apparatus involved and the surgical techniques, perfusates and conditions used. Not only is the information given in great detail, but the reasoning behind the choice of specific procedures is explained simply. All the questions the novice is likely to ask, such as those concerned with the importance of pH, the value of good temperature control and the need to counteract bacterial contamination, are answered in the same concise manner.

Having dealt with the practical aspects of the technique, the book discusses liver biochemistry. First the use of biochemical parameters to monitor the health and functioning of the tissue is examined, for example by considering concentrations of substrates and marker enzymes. The quantitative data here will be particularly welcomed by those new to the techniques. Later the use of the perfused liver in studies of biochemical mechanisms is discussed, using examples such as amino acid metabolism and glycogenesis.

Finally there are papers that report the use of liver perfusion for the study of drug and hormone metabolism. These indicate the potential of the technique, but if the book has any fault, it is that it fails to be critical here. Anyone who has used the techniques it recommends will be aware that, just as liver slices, homogenates or cell fractions are sometimes unsuitable preparations for a particular problem, the same can be true of the perfused liver. It is a pity that one of the experienced groups of contributors was not persuaded to summarize some of the difficulties and disadvantages of liver perfusion. However, since the book resulted from only the first European Meeting on the Technique of Liver Perfusion, there will perhaps be subsequent volumes in which this gap may be filled.

The Physiological Clock. Circadian Rhythms and Biological Chronometry. By E. Bünning. Revised 3rd ed. The English Universities Press Ltd., London; Springer-Verlag, New York, 1973. pp. 258. DM17.40.

Ever since Columbus regularly observed flickering lights from *Santa Maria* at precisely the same hour and moon phase, a phenomenon since ascribed to the luminous stream of

gametes discharged by the Bermudan fireworm, man has recorded his interest in the innate ability of living organisms to measure periods of time with computer-like accuracy. Even so, our understanding of the timing order of the environment in which we live still lags far behind our knowledge of the way in which organisms adapt themselves to the spatial organization around them. Nobody, however, can have made a greater contribution towards restoring the balance than the author of this volume, whose own research efforts in the field of biological rhythms have spanned a period of nearly half a century. Herein lies the great value of this third English edition of *Die physiologische Uhr*—that it has been written by a single author with an extensive knowledge of his subject. It has, moreover, been extensively revised to include data from the most important of the 600 or so papers on this subject published annually since the second edition appeared.

The title *Physiological Clock* sounds, in one sense, curiously obsolete, since our growing comprehension of circadian rhythms is gradually eroding some of the long-established concepts of physiology. In particular, the idea that life processes can be completely defined as merely the summation of the functions of individual organs is not compatible with the concept of a biological clock embracing the whole organism. Even Claude Bernard's idea of the "milieu interieur" can now be viewed less dogmatically when we consider that the whole spectrum of biological activities is really in a state of dynamic equilibrium dictated partly by endogenous diurnal rhythms and partly by the diurnal light regimen. The importance of these findings is evident in both social and medical contexts, ranging from a tendency to moodiness in shift-workers to the advisability of administering insulin to diabetics at the correct hour.

Judging by the contents of Dr. Bünning's latest volume, it is now difficult to conceive a level of organization not possessing its own innate circadian (from *circa diem*—about a day) rhythm, which facilitates the measurement of time in the same way as a visual sense estimates spatial dimensions. Circadian changes in sexual activity, for example, have been observed in unicellular algae, as well as in animals and man. The fact that isolated leaf sections exhibit diurnal fluctuations in turgor pressure and growth illustrates the principle that the biological clock does not necessarily depend on the interaction of the different components of an organism. Recent work in annelids, insects and vertebrates has shown that certain rhythms are maintained in isolated organs without the involvement of the brain. In the same way that decapitation fails to prevent the rhythmic contraction and expansion of annelid chromatophores, the time at which the vertebrate adrenal gland is removed influences the *in vitro* production of corticosterone by the adrenal cortex after its stimulation with adrenocorticotropic hormone. Despite this autonomy, however, some organs do exert a strong controlling effect and can synchronize processes in other parts of the body. The relative interdependence of the various behavioural and organ rhythms and the nature of the interaction between endogenous rhythms and those of terrestrial phenomena, notably tidal, lunar and seasonal rhythms, form a significant part of Dr. Bünning's book. This affinity between man and nature may not be exactly what Wordsworth conceived, but it does emphasize our total dependence on the world around us.

In an analysis of current ideas about the biochemical basis of biological rhythms, the author decides against the hypothesis that the clock function is strictly dependent on RNA synthesis, and suggests a more satisfactory hypothesis based on membrane phenomena. On the mathematical front, a simple model that can adequately describe circadian oscillations has yet to be formulated. Discussing the health implications of disturbed circadian rhythms, Dr. Bünning refers to the differing rates with which various body rhythms adjust

themselves to a phase-shifted light/dark cycle. Thus, circadian rhythms of gastric and intestinal hormone secretion are known, and asynchrony among the rhythms of stomach and liver can induce gastric ulcers.

In all, this latest volume, carrying some 1200 useful references, is an essential for all biologists whose research interests encompass periodicity in animal behaviour. The comprehensive and organized subject matter suggests that the book is not aimed primarily at the general reader.

Writing Medical Papers—A Practical Guide. By J. Calnan and A. Barabas. William Heinemann Medical Books Ltd., London, 1973. pp. vii + 121. £1.25

If the temptation to quote from a work is any measure of its success, this little book should prove very successful indeed. It is a rich source of phrases that might with benefit be displayed on the walls of Editorial Departments, and indeed of other offices and laboratories, in any scientific establishment. For while the authors have addressed themselves specifically to the medical profession, the greater part of their advice is equally applicable to those involved in other scientific disciplines. If one has any quarrel with this book it must surely be that the title is misleading, but this is a carping criticism, since the reader gets more rather than less than he is led to expect. The writing of medical papers has in fact been interpreted liberally to include such diverse literary activities as answering examination papers, applying for jobs, submitting applications for research grants, reviewing books and writing theses. Within this framework there is much practical advice, ranging from mundane instructions on the technique of correcting proofs to more entertaining suggestions regarding an author's possible responses to the rejection of his paper and the calculation of the "fog index".

In considering the craft of writing, the authors suggest a detailed plan of campaign covering each step from the collection of data to the final revision and submission of the manuscript. Guidance on the overall structure of the paper is backed by useful hints on common errors of punctuation and ways of achieving clarity and maintaining the readers' interest. There are also suggestions for further reading. The authors are forthright in their judgements—"the cardinal sin is vagueness, not bad grammar"—and stress the importance of their subject with the sobering adage that writing "may determine your own professional immortality".

Within the limits of mortal existence, however, perusal of this little book should help scientists who are new to the writing game and those who persistently find writing the least attractive aspect of their profession. While more experienced and enthusiastic writers may not agree with all the authors' suggestions, they too will find useful tips here and may be encouraged to take a fresh look at their own established methods and attitudes, an exercise that can hardly fail to be of benefit even to the most skilful writer. This handy guide should help us all to keep the authors' tenth commandment to "do better next time".

BOOKS RECEIVED FOR REVIEW

Nutritional Problems in a Changing World. Edited by Dorothy Hollingsworth and Margaret Russell. Applied Science Publishers Ltd., London, 1973. pp. xvi + 309. £10.00.

Progress in Toxicology. Special Topics. Vol. 1. By G. Zbinden. Springer-Verlag, Berlin, 1973. pp. 88. $4.70.

Datensammlung zur Toxikologie der Herbizide. Deutsche Forschungsgemeinschaft —Kommission für Pflanzenschutz-, Pflanzenbehandlungs- und Vorratsschutzmittel. Series 1. Verlag Chemie GmbH, Weinheim, 1974. pp. 152. DM58.

Chemical Mutagens. Vol. 3. Principles and Methods for their Detection. Edited by A. Hollaender. Plenum Publishing Co. Ltd., London, 1973. pp. xxii + 304. £10.35.

Residue Reviews. Residues of Pesticides and Other Contaminants in the Total Environment. Vol. 48. Edited by F. A. Gunther. Springer-Verlag, Berlin, 1973. pp. vii + 168. DM38.20.

Residue Reviews. Residues of Pesticides and Other Contaminants in the Total Environment. Vol. 49. Edited by F. A. Gunther. Springer-Verlag, Berlin, 1973. pp. vii + 158. DM32.90.

Microsomes and Drug Oxidations—Second International Symposium. Edited by R. W. Estabrook, J. R. Gillette and K. C. Leibman. The Williams & Wilkins Company, Baltimore, 1973. pp. viii + 486. $30.00.

The Striated Muscle. Edited by C. M. Pearson and F. K. Mostofi. The Williams & Wilkins Company, Baltimore, 1973. pp. xiii + 518. $32.00.

Environment and Birth Defects. By J. G. Wilson. Academic Press Inc., New York, 1974. pp. xiv + 305. £8.95.

Fd Cosmet. Toxicol. Vol. 12 pp. 757–776. Pergamon Press 1974. Printed in Great Britain

Information Section

ARTICLES OF GENERAL INTEREST

MORE FACETS OF FLUORIDE

The vexed question of where the hazards of fluoride begin and the benefits end continues to concern those who are trying to find a way through the maze of contradictory conclusions reached in reports on fluoridation and its consequences for affected communities.

New human studies

Krishnamachari & Krishnaswamy (*Lancet* 1973, **ii**, 877) have described severe ill-effects in 24 males from Andhra Pradesh, an area where fluorosis is endemic and the drinking-water contains 3·5–6 ppm fluoride. These patients, who were aged between 8 and 40 years, showed advanced dental changes and striking genu valgum, which produced a slow and laborious gait. In severe cases, flexion deformities of the knees interfered seriously with posture and movement. The earliest evidence of bony deformities appeared during early school age, and by 15 years the syndrome was fully developed. X-ray examination showed osteosclerosis of the spine in all but two patients, and in all of them sclerosis of the pelvis, humerus, scapulae, ribs and radius and ulna was present, with calcification of muscular attachments. The most striking radiological finding was osteoporosis affecting the lower end of the femur and upper ends of the tibia and fibula, with associated rarefaction of the metacarpal bones. Despite these findings, serum levels of calcium, phosphorus and alkaline phosphatase (AlkPase) remained within normal limits.

Krishnamachari & Krishnaswamy (*loc. cit.*) emphasized that these patients had a low dietary intake of calcium. Moreover sorghum, the staple diet of the area, contains a high proportion of phytate, which depresses calcium absorption from the gut. These factors may well aggravate the metabolic effects of a high fluoride intake, but since the syndrome has been observed predominantly in males, the possible role of hormonal factors in its pathogenesis cannot be ruled out.

An Editorial commenting on the Andhra Pradesh report (*Lancet* 1973, **ii**, 889) looked again at the papers of Leone *et al.* (*Publ. Hlth Rep. Wash.* 1954, **69**, 925; *Am. J. Roentg.* 1955, **74**, 874) pointing out the lack of crippling skeletal deformities in Bartlett, Texas, where until 1952 the drinking-water contained 8 ppm fluoride. It also referred to one of the controversial North Dakota reports (Bernstein *et al. J. Am. med. Ass.* 1966, **198**, 499), which described a higher incidence of osteoporosis among women drinking water containing 0·15–0·3 ppm fluoride than among a second group whose drinking-water contained 4–5·8 ppm fluoride. The Editorial concludes from these and other reports that a high fluoride intake cannot be the only major factor responsible for the Andhra Pradesh deformities. We have already noted the contradictory evidence from North Dakota (*Cited in F.C.T.* 1973, **11**, 1133).

This *Lancet* Editorial has not lacked critics. Gotzsche (*Lancet* 1973, **ii**, 1025) argues that many of the regional studies so far published have been statistically unsound, and that

the total amount of fluoride derived by individuals from various environmental sources is virtually uncontrollable, since the concentration in water does not indicate with any degree of accuracy an individual's actual daily intake. Further comments by Cook (*ibid* 1973, **ii,** 1026), underlining the contradictory nature of figures derived from areas of high and low natural fluoride levels, include the criticism that the actual daily intake of fluoride must replace the vague figure of drinking-water concentration before any conclusion regarding the optimal intake of fluoride can be accepted. Allen (*ibid* 1973, **ii,** 1148) argues for the safety of existing fluoridation schemes, on the basis that no substantiated ill-effects have been reported after fluoridation of public water supplies for 25 years in the USA and for 11 years in the UK.

Hyperparathyroidism occurring as a secondary effect of skeletal fluorosis has been reported by Teotia & Teotia (*Br. med. J.* 1973, **1,** 637). Their investigation of 20 patients, aged 42–68 years, with proven endemic skeletal fluorosis showed that five of them were in a hyperparathyroid state. The water in their local well contained 10·3–13·5 ppm fluoride and their mean daily intake from this source alone was estimated as 25 mg fluoride. The parathyroid hyperactivity associated with decreased solubility of bone fluoroapatite that occurs in skeletal fluorosis suggests a compensatory alteration to maintain a normal extracellular ionized-calcium equilibrium. It seems probable that fluoride may induce secondary hyperparathyroidism either by accelerating the crystal growth of fluoroapatite or by accelerating ion exchange between bone and serum. A direct action of excess fluoride upon the parathyroid gland cannot be excluded, however, without further study.

A double-blind clinical trial on 460 patients has been carried out in Finland (Inkovaara *et al. ibid* 1973, **1,** 613) to determine whether senile osteoporosis could be averted by prophylactic administration of the equivalent of 25 mg fluoride daily in the form of sodium monofluorophosphate. After treatment of the patients for 5 months, a high concentration of ionized fluoride was found in the plasma and the incidence of spontaneous bone fractures increased, so that the fluoride dose had to be reduced to 25 mg given twice weekly. Although no direct evidence emerged that this increase in liability to fracture was a direct result of fluoride retention, the need for further investigation into the relationship between raised plasma levels of fluoride and osteoporosis was evident. The value of studies on the possible protection offered by concurrent calcium and vitamin D therapy, perhaps with fluoride rather than monofluorophosphate treatment, was also indicated.

Allergy to fluoride has been authenticated, although it must be exceedingly rare. Ayres (*J. Am. med. Ass.* 1971, **218,** 1301) calls attention to a report (Shea *et al. Ann. Allergy* 1967, **25,** 388) of six children and one adult who developed allergic reactions after the use of toothpaste and vitamin preparations containing fluoride. Urticaria, exfoliative dermatitis, atopic dermatitis, stomatitis and gastro-intestinal and respiratory reactions were involved. A double-blind test in one patient and a patch test in another established the aetiological role of fluoride. Ayres (*loc. cit.*) observed, nevertheless, that these patients were exposed to far greater concentrations of fluoride than are used in the routine treatment of water.

Animal experiments

One of the complicating factors that may explain some of the conflicting evidence on the relationship between fluoride intake and osteoporosis is nutritional status. Experiments in cattle (Suttie & Faltin, *Am. J. vet. Res.* 1973, **34,** 479) indicate that nutritional inadequacy may increase the skeletal and dental lesions of fluorosis. From 14 weeks of age, heifers were fed 40 ppm fluoride, as sodium fluoride, with either 100 or 60% of the

recommended intake of total digestible nutrients, for 58 months. Although skeletal lesions in both groups were very mild, the group on the inferior diet showed a terminal increase in skeletal retention of fluoride compared with the other group. Dental fluorosis was more marked in animals on the reduced diet, and in spite of a somewhat lower intake of fluoride in this group, more fluoride appeared to be available for tissue storage. Undernutrition may therefore enhance the deleterious effects of excess fluoride.

Further studies on the dental concentrations of fluoride, citrate and carbonate have been reported by Kraak *et al.* (*J. Periodont.* 1972, **43,** 481), who fed cows on rations containing 12–93 ppm fluoride from the time they were 3–4 months old until they were 7·5 years old. The constituent layers of the mandibular second molar from each cow were separated and analysed. Fluoride concentrations in dental tissues were directly related to fluoride levels in the diet, and the uptake of fluoride by coronal and root cementum was markedly higher than that by enamel or dentine at a comparable level of fluoride exposure. There was a trend towards an inverse relationship between fluoride and citrate levels in all four dental tissues with dietary levels of fluoride up to 49 ppm, but the citrate content levelled off or rose in relation to fluoride in three of the four tissues at a dietary level of 93 ppm fluoride. These observations are consistent with the hypothesis that excess fluoride increases apatite-crystal size and limits citrate accumulation by decreasing the specific surface area. Carbonate and fluoride levels did not correlate in any dental tissue.

In studies on growing and adult rabbits, Rosenquist (*Acta path. microbiol. scand.* (*A*) 1973, **81,** 630) demonstrated a linear increase in the fluoride levels of cortical diaphyseal bone from the femur and tibia of animals given 0·5 or 10 mg fluoride/kg body weight/day for 2–14 weeks. No decrease in the fluoride content ensued when the fluoride supplement was withdrawn after administration for 14 weeks, by which time the rats were 21 weeks old and growth was almost complete. This indicated that the fluoride was firmly bound in the bone minerals and would only be lost in adult animals as a result of skeletal remodelling. The bone morphology of the rabbits given 0·5 mg fluoride/kg/day did not differ from that of the control animals, but the higher dose level was associated with excessive periosteal bone formation in the lateral part of the tibial cortex (*idem, ibid* 1973, **81,** 751). When fluoride treatment was terminated, the periosteal growth of this fluorotic trabecular bone ceased and there was a marked decrease in the porosity of the bone, in spite of the persistence of a high fluoride content in the bone ash. In a related series of experiments, Rosenquist (*ibid* 1973, **81,** 645) found that ingestion of 0·2 or 10 mg fluoride/kg/day between 7 and 21 weeks of age had no effect on serum AlkPase levels in the rabbit. With regard to the role of the parathyroid in fluorosis, studies of parathyroid activity in rabbits given fluoride in doses of 10 mg/kg/day for 14 weeks, as described above, did not support the hypothesis that excessive bone resorption in skeletal fluorosis was due to the secondary development of hyperparathyroidism (Rosenquist & Boquist, *ibid* 1973, **81,** 637). These authors failed to find any clear evidence of increased parathyroid activity in the treated animals.

The calcium content of the diet has an important bearing on fluoride toxicity. In some experiments reported by Srirangareddy & Rao (*Indian J. med. Res.* 1972, **60,** 481) rats on a normal or calcium-depleted diet were fed sodium fluoride equivalent to a dietary level of 45 ppm fluoride for 7 or 14 weeks, after which the level was raised to 135 ppm fluoride for 27 weeks. Food intake and growth in the fluoride-treated groups were not significantly different from those in the control groups, and serum calcium, phosphorus and AlkPase were not significantly affected. However, femur weights and density and the ash, calcium

and phosphorus levels of the bone were significantly reduced in rats on the low-calcium diet, a decrease which was not affected by the addition of fluoride to the diet for 7 weeks. Femur characteristics in rats on the full diet were not altered by the feeding of 135 ppm fluoride for 41 weeks, but in those on a reduced calcium intake, the ash, calcium and phosphorus contents of the femur rose. Osteoporosis was radiologically detectable in all animals fed a low-calcium diet, with or without fluoride.

We know that fluoride crosses the placenta and selectively deposits in foetal bone and developing teeth (*Cited in F.C.T.* 1973, **11**, 1131). Katz & Stookey (*J. dent. Res.* 1973, **52**, 206) have shown that the form in which fluoride is administered may alter its concentration in the foetus. The equivalent of 25 ppm fluoride was given to rats in drinking-water in the form of various salts during gestation, or during gestation and for 120 days prior to it, or only during the pre-gestational 120 days, and pups from each litter were examined. With sodium fluoride, stannous fluoride or stannous zirconium fluoride, the average fluoride content of each pup was 5·09–5·77 μg, compared with 1·58 μg in controls on a low-fluoride diet. With zirconyl hexafluorogermanate the average fluoride content of the pups rose to 7·29 μg. When fluoride was fed to rats for 120 days prior to gestation and withheld during gestation, transference to the foetus still took place, probably by mobilization from the maternal skeletal stores.

Higher levels of fluoride in the drinking-water (100–200 ppm), given to female mice maintained on a low-fluoride diet, retarded growth and impaired reproduction (Messer *et al. J. Nutr.* 1973, **103**, 1319). The high toxicity of the highest dose level was evidenced by a 50% mortality in 5 weeks. Growth and reproduction were not significantly affected in mice given 50 ppm in the drinking-water together with the low-fluoride (0·1–0·3 ppm) diet.

It has been suggested that the defect in urinary concentrating ability sometimes seen in patients after methoxyflurane anaesthesia may result from the inorganic fluoride released into the circulation by metabolism of the anaesthetic. A study of this problem in dogs has been reported by Frascino (*J. Lab. clin. Med.* 1972, **79**, 192). Intravenous infusion of 4–5 mg fluoride/minute in dogs increased the urinary flow rate by 20–40% and reduced tubular free-water reabsorption by 25–74%, while the glomerular filtration rate and sodium excretion rate remained stable. The depletion of maximal urinary concentration and tubular free-water reabsorption appeared to be related in degree to the blood level of fluoride rather than to the duration of exposure to it. Since fluoride failed to impair free-water clearance, it is not likely that it acts by interfering with sodium reabsorption in the loop of Henle. It seems possible, however, that any individual inability to excrete fluoride freely (*Cited in F.C.T.* 1973, **11**, 1134) might set up a cycle of deteriorating renal function in a patient subjected to an anaesthetic of which fluoride is a normal metabolite.

[P. Cooper—BIBRA]

BERYLLIUM TOXICITY—THE UNDERLYING PHENOMENA

A glance through the reviews of the biological properties of beryllium (Be) published over the last decade reveals that the element has two distinct types of toxicological effect. Acute exposure to Be compounds can produce cell death in organs in which Be accumu-

lates, whereas long-term exposure may induce malignant tumours. It is also evident that, under various conditions, Be compounds can exhibit a wide range of toxic effects, including hepatic necrosis, neoplastic changes and granular deposition in lysosomes.

Knowledge of the mechanism(s) underlying these effects is extremely limited. Some workers have suggested that the known affinity of Be for the nuclei of a variety of cells could be responsible for the range of toxic responses. More specifically, it has been shown that in regenerating rat liver Be inhibits enzymes involved in DNA synthesis (Witschi, *Biochem. J.* 1970, **120,** 623). In the same laboratory, Witschi & Marchand (*Toxic. appl. Pharmac.* 1971, **20,** 565) have extended these studies to other test systems, in which microsomal enzymes were induced selectively. Injection of rats iv with 50 μmols Be/kg body weight 24 hours before decapitation was followed by ip administration of either hydrocortisone acetate (150 mg/kg 5 hours before death), tryptophan (2 g/kg, 6 and 3 hours before death), 3-methylcholanthrene (50 mg/kg, 24 hours before death) or sodium phenobarbitone (75 mg/kg, 24 hours before death). Liver enzyme assays carried out at death revealed an inhibition of the induction of all the enzymes studied (tryptophan pyrrolase, acetanilide hydroxylase and aminopyrine demethylase). With a dose of 30 μmols Be/kg, inhibition of enzyme induction was also demonstrable when the animals were killed 72 hours after Be administration, except in the case of tryptophan pyrrolase induction by tryptophan. Dose-response relationships were established for hydrocortisone-induced tryptophan pyrrolase and for 3-methylcholanthrene-induced acetanilide hydroxylase. The authors concluded that the effects on enzyme induction were not due merely to a general hepatotoxic action of the element, but reflected a specific aspect of Be toxicology.

Early results obtained by Witschi (*loc. cit.*) tended to suggest that Be inhibited enzyme induction only if administered very early after partial hepatectomy, and implied that Be possibly interfered directly with the transcription of DNA. More recent work in the same laboratory (Marcotte & Witschi, *Res. Commun. chem. Path. Pharmac.* 1972, **3,** 97) has raised doubts about this postulate, since the rate of hepatic RNA synthesis appeared to be unaffected by Be administration. Rats were partially hepatectomized and immediately afterwards were given 30 μmols Be/kg iv, followed at once or 2, 4 or 6 hours later by an injection of [^{14}C]orotic acid. Exactly 20 minutes after the orotic acid injection, incorporation of the label into total cellular RNA (an index of nuclear RNA synthesis) was determined. In a further experiment, animals were killed 4 or 12 hours after the [^{14}C]orotic acid injection, and labelling of cytoplasmic RNA was measured to detect any changes in the cytoplasmic RNA labelling pattern that might indicate whether Be had any effect on RNA transfer from the nucleus or on RNA degradation. In both experiments, RNA labelling in Be-treated animals was similar to that observed in control rats, and nuclear RNA polymerase activity was essentially the same in both groups.

To investigate any qualitative changes in RNA metabolism, cytoplasmic RNA was examined on sucrose density gradients at various intervals after partial liver removal. Again no difference between Be-treated and control animals was noted. Neither was there any obvious Be-induced disturbance of [^{14}C]leucine incorporation into acidic nuclear protein or of histone acetylation in the nucleus. The theoretical explanation offered by the authors for the apparent inability of Be to affect nuclear protein metabolism involves the possible regulation of transcription through interaction of divalent cations with chromatin. They suggest that Be may replace magnesium in the chromatin and then block further aggregation and dispersion of this genetic material, but evidence is not yet available to support this theory.

Morphological examination of the subcellular effects of Be compounds on regenerating liver have recently been conducted by Goldblatt *et al.* (*Archs envir. Hlth* 1973, **26**, 48). In light-microscopic studies, partially hepatectomized male rats were injected iv, immediately after surgery, with 15, 30 or 60 μmols Be sulphate/kg and examined at 6, 12, 24 or 48 hours after injection, or alternatively were injected with 30 μmols Be sulphate/kg 8, 12 or 22 hours after partial hepatectomy and were killed at 28 hours. Liver necrosis only became evident 36–48 hours after Be treatment, during which period most rats given the higher doses died. Before this, however, mitosis was inhibited in the regenerating liver, being completely absent in animals treated with Be 16 or 20 hours before death. Under the electron microscope, hepatic parenchymal cells of both intact and partially hepatectomized animals examined 24 hours after Be injection showed vacuolization and dense deposits in the lysosomes, loss of fibrils and dense plaque formation in some nucleoli and changes in smooth endoplasmic reticulum and Golgi complexes. Complex structural alterations were also evident in the bile canaliculi. These morphological observations correlate well with the biochemical studies mentioned earlier. In particular, the reported inhibition of DNA synthesis can be related to the great decrease in mitotic figures some 24 hours after Be sulphate administration. Similarly, a marked release of acid phosphatase from lysosomes has been reported 24 hours after Be administration, and an enlargement of lysosomes like that observed in this microscopic study is often associated with a shift in sedimentation of acid phosphatase.

As an offshoot from the Be-provoked suppression of hepatic microsomal-enzyme synthesis, it has been suggested that Be might inhibit the induction of pulmonary mixed-function oxidases, particularly the aryl hydrocarbon hydroxylase (AHH) complex responsible for detoxification of the ubiquitous carcinogen, benzo[*a*]pyrene (BP). In a recent study (Jacques & Witschi, *ibid* 1973, **27**, 243), rats were injected intratracheally with a neutralized Be sulphate solution in a dose of 150 μmols/kg. Pulmonary AHH activity was measured 1, 2 and 7 days and 2 and 4 weeks after Be administration. Although the specific activity of the enzyme complex was lowered by Be, changes in total AHH activity per lung were much smaller and were significant only for 2 days after Be injection. RNA synthesis in the lungs of rats exposed to Be was higher than in controls, a difference ascribed to the acute pneumonitis elicited by the physico-chemical properties of the Be salt. Be failed to disturb the induction of pulmonary AHH by methylcholanthrene (50 mg/kg) administered ip 24 hours before the animals were killed. These results provide no support for the hypothesis that Be could act as a pulmonary co-carcinogen through interference with the metabolism of such carcinogens as BP.

There is evidence that not all the adverse effects attributed to Be are instigated at the biochemical level. The mechanism by which Be inhibits the activity of the reticulo-endothelial system (RES), for example, appears to be related more to the physico-chemical properties of Be compounds than to any cytotoxic action of these materials. In a recent investigation of this mechanism, Vacher *et al.* (*Toxic. appl. Pharmac.* 1973, **24**, 497) compared the acute iv toxicity and RES-depressing effect of a soluble Be salt (sulphate) and an insoluble Be salt (phosphate) with the effects of carbon. Such an experimental design was required to differentiate between the possible effects of Be phosphate aggregates and a diffusible complex of Be with plasma organic acids, both fractions being found in the blood after injection of Be sulphate. Although Be sulphate was five times more acutely toxic than the phosphate when injected iv into mice, the dose-response relationship was virtually identical for the two salts. It was apparent that the RES blocking effect was due

primarily to the diffusible Be phosphate aggregates which were phagocytozed by the cells of the RES. This effect could not be explained in terms of a cytotoxic action, since Be phosphate is suppressive at doses 100 times lower than those which evoke Be toxicity. Be-induced RES blockade was much more marked than that induced by carbon and proved more persistent. Since these two particle types have very different physico-chemical properties, the authors suggest that blockade occurs as a result of saturation of absorption sites on the phagocytic cells and ingestion of the occupied sites.

Physico-chemical factors may also be involved in the onset of occupational berylliosis, since it is probable that the pulmonary response to Be compounds is immunologically based. In addition to the hypersensitivity to these compounds that has been demonstrated by skin testing, workers susceptible to pulmonary disease in Be-contaminated environments often show hypergammaglobulinaemia (*Cited in F.C.T.* 1971, **9**, 573). The recent work of Conradi *et al.* (*Archs envir. Hlth* 1971, **23**, 348) involving examination of experimental animals for chronic pulmonary changes after exposure to atmospheres containing BeO (calcined at 1400°C) is of great relevance here. Five monkeys and six dogs of either sex were exposed for 30-minute periods three times at monthly intervals to an atmosphere containing an average of 3·3–4·38 mg Be/m^3. Two years after exposure, electron microscopy of lung tissue revealed deposits of unidentifiable particles within the lysosomes of histiocytes, endothelium and macrophages, in both experimental and control animals. Morphometric examination revealed no thickening of the air–blood barrier in exposed monkeys or dogs, nor any proliferative changes in this tissue mass. In dogs, vacuolated interstitial cells, thought to be degranulated mast cells, were observed to a comparable extent in the pulmonary tissue of test and control animals. Thus, while spectrographic analysis revealed that Be oxide was deposited in the lungs of both monkeys and dogs, microscopic examination indicated that Be did not evoke pathological changes in the lung within 2 years of its inhalation.

One of the difficulties in investigating the basis of chronic berylliosis is, in fact, the extremely long delay (10–15 years) sometimes observed in the onset of this disease. One hypothesis proposed to account for this latency involves a triggering mechanism called into play, perhaps by pregnancy, infection or surgery, to elicit an adrenocortical-hormone imbalance of the type that has frequently been known to precipitate the onset of beryllium disease. To simulate such hormonal imbalance during the early stages of berylliosis, Clary *et al.* (*Toxic. appl. Pharmac.* 1972, **23**, 365) treated mice and guinea-pigs with metyrapone (2-methyl-1,2-di-3-pyridyl-1,1-propanone), which acts by inhibiting 11-β-hydroxylation in corticosteroid biosynthesis. The animals received labelled Be in the form of ^{7}Be sulphate or ^{7}Be oxide by transthoracic or intratracheal injection and a month later metyrapone was administered sc. After another month the animals were sacrificed and the Be content of lung, liver, spleen, kidney, heart, adrenal and femur was determined.

In both species, alteration of adrenal function was accompanied by a decrease in the skeletal concentration of Be, and an increase in the Be content of the liver, although the latter level subsequently decreased again. The fact that such changes in metyrapone-treated animals were more marked in males suggested a possible sex difference in Be mobilization. Animals with altered adrenal function were also more affected by Be treatment than control groups, as indicated by weight loss, metal-ion shift and increased levels of serum isocitrate dehydrogenase and alkaline phosphatase. The results presented in this paper thus offer a working hypothesis to account for the long latency often associated with berylliosis. It is conceivable that, under stress conditions, intracellularly located Be could

be activated to prevent the induction of enzymes in various organs. Further investigation of the influence of Be on enzyme systems where adrenal function has been modified would clearly be of interest.

[J. J.-P. Drake—BIBRA]

THE MOUSE AND DDT

The perennial question of the possible carcinogenicity of DDT has given rise to a considerable amount of toxicological effort as well as a great deal of emotional reaction. Past evidence suggesting a definite, though slight, carcinogenic potential has tended to be confused and far from convincing and this prompted several groups to undertake further studies, a course of action backed by a recommendation from FAO/WHO. Reports on some of these studies are now available, including those from parallel studies carried out on mice by the International Agency for Research on Cancer, Lyon, and the Istituto Nazionale per lo Studio e la Cura dei Tumori in Milan.

Some results from one of these, a multigeneration feeding study in which dimethylnitrosamine (DMNA) was used as a positive control, have been reported by Terracini *et al.* (*Int. J. Cancer* 1973, **11**, 747). The report is limited to the findings in the first two generations of BALB/c mice given 2, 20 or 250 ppm technical DDT in the diet or 0·0003% DMNA in the drinking-water, starting from the time of weaning. Poor survival rates, due largely to fighting, limited the evaluation possibilities in males, but 250 ppm DDT appeared to induce liver-cell tumours in a high proportion of mice of both sexes. In the females, liver tumours appeared in 44% of the parent (F_0) group and 74% of the first (F_1) generation given 250 ppm DDT. These tumours did not metastasize, but they grew when transplanted into syngeneic mice. No liver tumours appeared in the groups fed lower concentrations of DDT or in the control groups. In contrast, malignant lymphomas arose in about half of the mice fed 2 or 20 ppm DDT or a DDT-free diet, while at the 250 ppm level of DDT malignant lymphomas were induced in only 14% of one colony and 36% of a second. Feeding of DDT was not associated with any increased incidence of lung adenoma. Tumours in sites other than the liver, lungs and lymphatics were fewer in mice fed DDT than in the controls, particularly in the F_0 generation, although no linear dose-response relationship could be established. Addition of DMNA to the drinking-water consistently induced lung adenomas, hepatic blood cysts and haemangioendotheliomas. The results of the study suggest that in the mouse the liver is the only target organ for DDT carcinogenicity and that the threshold for carcinogenicity lies between 20 and 250 ppm DDT, levels equivalent to daily intakes of 3 and 37·5 mg/kg, respectively. The earlier appearance of liver-cell tumours in the F_1 than in the F_0 generation was possibly a reflection of the foetal and neonatal exposure to DDT, but this has not been definitely established.

This work supports the interim findings of liver-tumour induction by DDT demonstrated in a parallel study in CF-1 mice (*Cited in F.C.T.* 1973, **11**, 911). The latter study has now been completed and reported in full (Turusov *et al. J. Natn. Cancer Inst.* 1973, **51**, 983). Technical DDT was fed to CF-1 mice at dietary levels of 2, 10, 50 or 250 ppm over six successive generations (F_0 and F_1–F_5). In males, the incidence of liver tumours rose from 30% in the control group to 50–56% in the three lower dosage groups and to 86% in the group given 250 ppm DDT. In females, however, the increase to 66% in the

group fed 250 ppm DDT was the only significant rise over the control value of 5%, and 2 ppm DDT had no effect. The mean lifespan of males with hepatoma fell from 114 weeks in controls to 84 weeks in the 250 ppm DDT group, but in females the reduction (from 104 to 94 weeks) was not significant. The incidence of hepatomas did not increase with successive generations and DDT had no significant effect on the incidence of tumours at sites other than the liver, although there appeared to be a slight increase in lung tumours in mice given the 2 or 10 ppm DDT diet. Malignant liver tumours, tentatively termed hepatoblastomas, showed a significant increase in the 250 ppm DDT group and a marked tendency to metastasize to the lungs.

A rather different picture emerges from a study reported by Shabad *et al.* (*Int. J. Cancer* 1973, **11**, 688) involving the daily administration of technical DDT in sunflower-seed oil to A-strain mice by gastric intubation. Daily doses of 10 mg DDT/kg were given to a parent (F_0) generation and to five succeeding generations (F_1–F_5), but the higher dose given (50 mg/kg) had a generalized toxic effect on the F_0 generation and prevented the breeding of further generations. Dosing was started in each group 2–4 weeks after weaning and was continued until the animals were killed when 1 year old. The only tumours reported in these animals were lung adenomas, the incidence of which increased to 37% in the group given 50 mg DDT/kg daily and to 20% in the F_0 generation given 10 mg/kg, compared with 7% in the controls. In the F_1–F_5 generations, the lung-tumour incidences were 15, 24, 46, 43 and 13%, respectively. The authors suggest that the absence of other tumours from these mice, and particularly the freedom from the liver tumours found in the study on CF-1 mice already mentioned, were probably due partly to inter-strain differences in susceptibility and partly to the relatively restricted length of this study.

In order to throw some light upon the selectivity of DDT-induced tumours, Tomatis *et al.* (*Tumori* 1971, **57**, 377) studied the distribution of DDT and its metabolites in CF-1 and BALB/c mice fed 2, 20, 50 or 250 ppm technical DDT over six generations. The highest concentrations of DDT and metabolites occurred in fatty tissues, followed in descending order by the reproductive organs, liver, kidney and brain. *p,p'*-DDT was the most prevalent metabolite in organs other than the liver, where *p,p'*-DDD showed the highest concentration. All metabolites showed lower concentrations in pregnant than in non-pregnant females. DDT residues in the foetuses were directly proportional to the concentration of DDT in maternal feed. Concentrations of *p,p'*-DDT and *p,p'*-DDD in foetuses and placentas of the same litter showed a strong negative correlation, while *p,p'*-DDE accounted for only 5% of the total foetal and placental residues. Shortly after birth, the whole-body concentration of DDT showed a significant increase, probably on account of the high intake of DDT in milk from the treated mothers.

These studies add little to the already published data, but one point encourages some speculation. If one accepts as a negative result the finding that administration of high levels of DDT to successive generations did not increase tumour incidence or the incidence of obvious malformations, some reassurance may perhaps be gained that DDT stored in body tissues may not constitute as great a genetic hazard as has sometimes been suggested.

[P. Cooper—BIBRA]

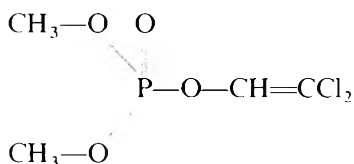
STUDIES ON DICHLORVOS

Introduction

From time to time we have referred to isolated studies on the safety and use of the organophosphorus pesticide, dichlorvos (*Cited in F.C.T.* 1969, **7**, 82; *ibid* 1970, **8**, 461; *ibid*

1971, **9,** 587 & 588). The past 3 years, however, have seen the publication of a large number of papers on this subject and the time seems opportune to take an overall look at the picture they present.

Dichlorvos is *O,O*-dimethyl-2,2-dichlorovinyl 1-phosphate (DDVP):



It is perhaps best known as the major active ingredient of slow-release resin strips used for the control of flying insects in domestic and other environments. Many devices of this kind are on sale throughout the world. In some, such as the Vapona® strip, the dichlorvos acts as a plasticizer in the polyvinyl chloride base, an arrangement which provides a sustained but slow release of dichlorvos and thus maintains an insecticidal concentration in relatively enclosed atmospheres for some months. Dichlorvos is active against a wide variety of pests, and appears in several different formulations. It is, for example, used in domestic aerosol sprays, in the insecticidal fumigation of stored grain, tobacco and other products, in pre- and post-harvest crop treatments and as an anthelmintic for farm animals and dogs. In the UK, approval has been given for the use of dichlorvos on glass-house crops and outdoor fruit and vegetables, and the dichlorvos strip is approved for use in a wide range of domestic situations, including kitchens. In the USA, the use of dichlorvos strips in domestic situations is similarly approved except that use in kitchens and restaurants has been restricted, apparently on the grounds that the continuous emission of any pesticide is an undesirable means of combatting the intermittent presence of insects in areas where food may be exposed. Both pre- and post-harvest applications to a number of agricultural crops are also registered in the USA, and tolerances in the 0·5–2 ppm range have been established for raw agricultural products. The 1970 Joint Meeting of the FAO Working Party of Experts and the WHO Expert Group on Pesticide Residues (AGP: 1970/M/12/1; WHO/Food Add./71.42, p. 164, Rome, 1971) also recommended a series of tolerances in agricultural commodities.

Like other organophosphorus compounds, dichlorvos owes its insecticidal capacity to its ability to inhibit the activity of the cholinesterase (ChE) enzymes, an effect which may also be produced in mammals under suitable conditions. Unlike some pesticides of this class, which require metabolic conversion to an active compound, the dichlorvos molecule itself exerts this antiChE effect.

Because of the use of dichlorvos in slow-release resin strips, which provide a condition of continuous exposure for anyone remaining within the treated environment, much of the work on the safety evaluation of this compound has been concerned with inhalation studies. In this connexion, however, it is important to remember that while a resin strip may contain about 20% dichlorvos, the slowness of its release into the atmosphere means that the toxic hazard presented by the strip is very different from that of the technical material. Nevertheless, inhalation and other routes of exposure cannot be discounted, even in connexion with the strip formulations. Skin contact is liable to occur occasionally during handling, while the question of oral toxicity is important not only because of the poss-

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ible presence of residues in foodstuffs but also because the strip formulations could be chewed by infants—as has in fact occurred from time to time.

Oral toxicity

Acute studies on dichlorvos have demonstrated the marked ChE-inhibiting properties of high doses. Mortality was 25% in dogs given technical-grade dichlorvos in a single oral dose of 22 mg/kg, all those treated showing a rapid depression of erythrocyte acetylChE and serum ChE (Snow & Watson, *Aust. vet. J.* 1973, **49**, 113). A reduction in brain ChE levels demonstrated the ability of dichlorvos to cross the blood–brain barrier. Other effects were an increase in haematocrit and some serum-enzyme changes possibly indicative of muscle damage. Similar effects followed iv injection of 2·2–11 mg/kg (Snow & Watson, *loc. cit.*), the dogs that died showing signs typical of organophosphorus intoxication. In mice, the intragastric LD_{50} of dichlorvos was about 140 mg/kg, while 500 ppm in the diet fed for 24 hours roughly halved serum ChE, which returned to normal in about 8 days (*Cited in F.C.T.* 1971, **9**, 587).

Foals given dichlorvos in a vegetable oil-based gel showed mildly toxic signs with marked ChE inhibition after a dose of 50 mg/kg and severe toxicosis after 400 mg/kg (20 times the therapeutic dose), but the return to normal was rapid in all cases (Albert & Stearns, *Am. J. vet. Res.* 1973, **34**, 1359). Rhesus monkeys given 21 daily doses of 20–80 mg/kg, as PVC pellets containing 20% dichlorvos, showed no overt signs of organophosphate poisoning, although inhibition of both plasma- and erythrocyte-ChE levels was virtually total (Hass *et al. J. Am. vet. med. Ass.* 1972, **161**, 714). At the end of the treatment, ChE activity returned to normal in about 3 weeks in the plasma and in 50–60 days in the erythrocytes. These studies were carried out in connexion with the oral use of dichlorvos formulations to combat parasitic-worm infections in animals, although they are obviously of wider interest.

Other evidence has been obtained directly in man, for whom, it seems, the strip formulations offer little hazard of acute poisoning. Several young children have chewed Vapona strips without developing any adverse effects, even in the absence of treatment (Gillett *et al. Residue Reviews* 1972, **44**, 115). In the longer term, plasma-ChE activity was reduced by 70% in adult volunteers fed 2·5 mg dichlorvos daily for 20 days, but erythrocyte ChE was not affected (*Cited in F.C.T.* 1969, **7**, 82). Some estimate of the relevance of such exposure to the amounts of dichlorvos ingested in foods exposed to dichlorvos strips may be gleaned from several analytical studies. Leary *et al.* (*Toxic. appl. Pharmac.* 1971, **19**, 379) reported average residues of 0·1 ppm in foodstuffs exposed to abnormally high concentrations of strips in treated homes, while lower concentrations, generally ranging from below 0·02 to 0·05 ppm, were found in other studies involving a more normal domestic use of the strips (Collins & DeVries, *Bull. env. contam. & Toxicol.* (*U.S.*) 1973, **9**, 227; Elgar *et al. Pestic. Sci.* 1972, **3**, 601).

Somewhat higher levels have been demonstrated in meals carried in aircraft sprayed with dichlorvos during flight to prevent the international transport of disease-carrying insects (Dale *et al. J. agric. Fd Chem.* 1973, **21**, 858). The highest contamination was found in margarine (up to about 1 ppm after 30 minutes in an atmosphere likely to occur in an aircraft cabin after disinsection) and it was calculated that consumption of a complete dinner under such conditions might lead to ingestion of 60 μg dichlorvos. This, of course, would be an isolated situation, different from the continuous condition pertaining in a

strip-treated home, and the study involved an unusually adverse situation regarding food exposure during spraying.

In the previously mentioned study of homes containing a high proportion of strips, Leary *et al.* (*loc. cit.*) found no significant effect on ChE levels or other parameters among the families living and eating in the houses in question. It is noteworthy that the acceptable daily intake of dichlorvos derived by FAO/WHO from oral studies is 4 μg/kg, or 240 μg for a 60-kg adult (*Cited in F.C.T.* 1968, **6**, 753).

Dermal toxicity

The effects of skin contact with dichlorvos have also been studied directly in man. Direct application of Vapona strips to the skin for 30 minutes on each of five successive days indicated that no acute hazard was involved in the handling of strips (Gillett *et al. loc. cit.*), while no effect on ChE levels was detected in infants who wore for 5 days garments that had been exposed to high levels of dichlorvos in an enclosed space and that initially contained 0·26–0·40 ppm of the insecticide.

Inhalation studies

Inhalation thus remains the most important route of entry of dichlorvos into the body. Recent contributions on this aspect have included tests on dogs, cats and rabbits exposed continuously for 8 weeks to dichlorvos atmospheres generated from impregnated strips (Walker *et al. Arch. Tox.* 1972, **30**, 1). No effects on general health or behaviour, plasma or erythrocyte ChE activity or electroencephalograms were found in any species, although exposure was continuous and the atmospheric concentrations produced under the test conditions were in the 0·05–0·3 μg/litre range and were therefore somewhat higher than those normally encountered in domestic use. In a study involving over 3000 air samples from houses containing Vapona strips used as recommended, 97% of the samples contained less than 0·1 μg dichlorvos/litre irrespective of the prevailing climate. Ranges found in UK samples were 0·03–0·06 μg/litre when strips had been in place for 1 week, 0·02–0·04 μg/litre at the end of 1 month and <0·01–0·02 μg/litre after 3 months (Elgar & Steer, *Pestic. Sci.* 1972, **3**, 591).

In an inhalation study in chickens (Rauws & Van Logten, *Toxicology* 1973, **1**, 29), plasma ChE was depressed significantly by continuous exposure to about 0·2 μg/litre air for 21 days or by 16-hour periods of similar exposure for 28 days. Exposure to about 0·1 μg/litre for 21 full days or to 0·2 μg/litre for 8-hour periods on 28 days had no effect. Brain acetylChE was depressed by the more severe continuous exposure. The depression of plasma ChE in chicks exposed for 16-hour periods was in contrast to the findings in man reported by Cavagna *et al.* (*Archs envir. Hlth* 1969, **19**, 112), who detected no effect on plasma or erythrocyte ChE in healthy subjects exposed to 0·28 μg/litre for daily periods of 16 hours. The plasma ChE of the chick has been shown *in vitro* to be about as sensitive to dichlorvos as is that of man, but the minute volume (blood volume passing through the lungs in 1 minute)/kg body weight is much higher in the chick than in man and the relative rates of dichlorvos metabolism in the two species are unknown. Nevertheless, as far as continuous 24 hours/day exposures are concerned, the results of the chick study are comparable with earlier findings in man (*Cited in F.C.T.* 1970, **8**, 461; *ibid* 1971, **9**, 588) suggesting a no-effect-level for continuous exposure of about 0·1 μg/litre. In fact, in man, a continuous 24-hour exposure for several days to some 0·25 μg/litre seemed to be needed to produce a significant decrease in plasma ChE. The results of these and other inhalation

studies have led to the setting of a US threshold limit value (TLV) of 1·0 μg/litre for industrial exposure (8 hours/day, 5 days/week).

In assessing safe conditions of exposure, particularly of slow-release formulations, account has to be taken of the fact that the sections of the population liable to be most sensitive to the pesticide (such as chronic invalids and newborn infants) are also those most likely to remain for prolonged periods in a treated atmosphere. However, it appears that of these 'captive targets' only those with impaired liver function have shown marked evidence of an increased sensitivity to dichlorvos in respect of their already diminished or diminishing plasma ChE (*ibid* 1970, **8,** 461; Vigliani, *Toxic. appl. Pharmac.* 1971, **19,** 379).

Reproduction and teratogenicity studies

Another part of the population requiring special consideration in the safety assessment of any environmental chemical is the developing foetus. In a toxicity screening study in rats, a large ip dose (15 mg/kg), which was toxic to the dams, produced some malformations when given on day 11 of pregnancy but had no effect on foetal resorptions or on foetal and placental weights (*Cited in F.C.T.* 1972, **10,** 411). Studies by Khera & Lyon (*Toxic. appl. Pharmac.* 1968, **13,** 1) in chick and duck eggs and by Vogin *et al.* (*ibid* 1971, **19,** 377) in orally dosed rabbits also indicated that effects on foetal development occurred only with doses that were markedly toxic. In three-generation feeding studies on dichlorvos in pigs (Collins *et al. ibid* 1971, **19,** 377) and rats (Witherup *et al. ibid* 1971, **19,** 377), no effects on reproductive parameters were detected with dietary levels up to 500 ppm. Finally, in an inhalation study, Thorpe *et al.* (*Arch. Tox.* 1972, **30,** 29) exposed groups of rabbits and rats throughout pregnancy to several different atmospheric concentrations of dichlorvos, ranging from 0·25 to 6·25 μg/litre. No evidence of any teratogenic effect was obtained even with the higher levels of exposure, which caused obvious signs of toxicity as well as depression of ChE in both species and which killed some of the rabbits.

Mutagenicity studies

Demonstrations of the ability of dichlorvos to alkylate nucleic acids *in vitro* (*Cited in F.C.T.* 1971, **9,** 587) have inevitably raised the question of the compound's possible mutagenicity or carcinogenicity, a question which has been reviewed in some detail by Bedford & Robinson (*Xenobiotica* 1972, **2,** 307). These authors discuss the reaction mechanisms involved in alkylation and, having determined second-order rate constants for the reaction at 37°C, compare the constants for dichlorvos and several other organophosphorus insecticides with those of several known biological alkylating agents. They then elaborate on various chemical and biochemical factors which may explain why the alkylating reactivity of dichlorvos demonstrated in the laboratory does not give rise to patently mutagenic or carcinogenic effects in mammalian systems.

The absence of mutagenic effects in higher organisms has been confirmed in several studies on dichlorvos, but attempts to demonstrate some mutagenic potential in microorganisms have been somewhat more successful. Ashwood-Smith *et al.* (*Nature, Lond.* 1972, **240,** 418) found that dichlorvos, applied in the form of Vapona strips to agar-plate cultures of an *Escherichia coli* mutant, increased the mutant yield by a factor of 3–6, compared with a factor of about 45 found with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine by the same method. A similar slight increase in mutation rate was recorded after incubation of *E. coli* with 0·1% dichlorvos (Voogd *et al. Mut. Res.* 1972, **16,** 413). In another *in vitro*

study, Dean (*Arch. Tox.* 1972, **30,** 67) showed that dichlorvos in high concentrations (25–100 mg/ml) in dimethylsulphoxide produced dose-related increases in the reverse mutation rate when applied to agar-plate cultures of *Serratia marcescens*. A saturated aqueous solution also increased the reversion rate in this organism, but dichlorvos added to cultures of *E. coli* in both saturated and dilute aqueous solution failed to induce reverse mutation, although positive results were obtained in this organism with methyl methanesulphonate (MMS), β-propiolactone, nitrogen mustard and other known chemical mutagens.

Bridges *et al.* (*Mut. Res.* 1973, **19,** 295) confirmed that dichlorvos and MMS showed similar patterns of mutagenic activity in *E. coli* WP2 deficient in various DNA repair pathways, but showed that MMS was by far the more active of the two. The results were consistent with the compounds' known ability to alkylate DNA. Evidence of a qualitative similarity and quantitative differences between these two compounds was also demonstrated by Wild (*ibid* 1973, **19,** 33) using streptomycin resistance in *E. coli* as the test system, while Rosenkranz (*Cancer Res.* 1973, **33,** 458) showed that, like MMS and certain other DNA-alkylating agents, dichlorvos was more cytotoxic to *E. coli* deficient in DNA polymerase than to normal *E. coli* cells. In another *in vitro* study (Dean, *Arch. Tox.* 1972, **30,** 75), dichlorvos added to cultures of human lymphocytes at various stages of the cell cycle produced no detectable chromosomal aberrations, although concentrations of 5–40 μg/ml were cytotoxic.

Efforts to relate the *in vitro* alkylating properties of dichlorvos directly to its activity in the mammal have made use of recently developed techniques for demonstrating mutagenicity. Carrying out a dominant lethal mutation test in mice, Dean & Thorpe (*ibid* 1972, **30,** 51) exposed groups of males to atmospheres containing 30 or 55 μg dichlorvos/litre for a single period of 16 hours or to concentrations of 2·1 or 5·8 μg/litre for 23 hours/day over a period of 4 weeks. For 8 weeks after the end of treatment each mouse was mated with a succession of untreated females, so that a complete spermatogenic cycle was spanned by the test matings. No increases in pre-implantation losses or early foetal deaths were found in the resulting pregnancies to indicate any mutagenic effect, and no impairment of fertility was detected in the treated males. As an extension of this study (*idem, ibid* 1972, **30,** 39), mice were exposed to air containing 64–72 μg dichlorvos/litre for 16 hours or to 5 μg/litre for 21 days, and hamsters were exposed to 32 μg/litre for 16 hours or given a single 15 mg/kg oral dose or two doses of 10 mg/kg. The incidence of chromosome abnormalities seen in preparations made from bone marrow and spermatocytes from the treated animals did not differ from that in preparations from control groups.

Another dominant lethal study covering 174 pesticides, food additives, industrial chemicals, known carcinogens, pharmaceuticals, air and water pollutants and various miscellaneous chemicals, revealed no significant mutagenic activity on the part of dichlorvos given in five oral doses of 5 or 10 mg/kg or an ip dose of 13·0 or 16·5 mg/kg (Epstein *et al. Toxic. appl. Pharmac.* 1972, **23,** 288).

An important development in mutagenicity studies has been the host-mediated assay, which combines exposure of the chemical to the metabolic processes of the host mammal with the advantages of demonstrating some form of gene mutation in simple microorganisms. Dean *et al.* (*Arch. Tox.* 1972, **30,** 61) used mice dosed orally with 50 or 100 mg dichlorvos/kg or exposed for 5 hours to atmospheres containing 60 or 99 μg/litre, and determined the rate of mitotic gene conversion in suspensions of yeast (*Saccharomyces cerevisiae*) injected ip into male mice immediately before the dichlorvos treatment and harvested again from the peritoneal cavity 5 hours later. No increase in mitotic gene conver-

sion occurred in the yeast cells harvested from any of the dichlorvos-treated mice, although in stationary-phase liquid cultures of the yeast exposed directly to dichlorvos in a concentration of 4 mg/ml, mitotic gene conversion was increased at both the susceptible loci. In contrast, a marked increase in conversions was seen in yeast cells harvested from positive-control mice treated with ethyl methanesulphonate. Although dichlorvos has been shown to have some effect on *S. marcescens in vitro*, an sc dose of 25 mg/kg had no effect on the mutation frequency of *S. marcescens* used for a host-mediated assay in mice (Buselmaier *et al. Biol. Zentbl.* 1972. **91,** 311).

Metabolism

The key to the negative results in these *in vivo* mutagenicity tests, and to the low toxicity of dichlorvos to mammals generally, lies in the compound's metabolic fate. We have already mentioned that the antiChE activity and other toxic effects of this organophosphorus insecticide reside in the parent compound, so that metabolism detoxicates rather than activates dichlorvos, and there is much evidence that this metabolism occurs very rapidly in mammals, irrespective of the route of entry into the body.

Although the metabolism of dichlorvos occurs in many tissues, the liver is the main site of detoxication (*Cited in F.C.T.* 1968, **6,** 395). Studies using [^{14}C]methyl-labelled dichlorvos showed that in mice and rats metabolism of an oral dose was almost complete in 24 hours (*ibid* 1973, **11,** 689), with some 60% of administered radioactivity appearing in the urine and 15% in exhaled air. Hydrolysis of the phosphate–vinyl bond and demethylation were major metabolic pathways, and the presence of small quantities of *S*-methylcysteine derivatives in the urine provided clear evidence of some involvement of glutathione in the dealkylation reaction. Oral administration of [^{14}C]vinyl-labelled dichlorvos to the rat, mouse, hamster and man confirmed the rapidity of metabolism in all four species (Hutson *et al. Xenobiotica* 1971, **1,** 593; Hutson & Hoadley, *Arch. Tox.* 1972, **30,** 9). Much of the radioactivity was expired as carbon dioxide, but a significant amount was retained in the body some 4 days after treatment because of its incorporation into intermediate metabolic pathways. It is postulated that the demonstrated incorporation of [^{14}C]glycine and [^{14}C]serine into liver protein in the rat resulted from conversion of dichlorvos via dichloroacetaldehyde and glyoxylate (Hutson *et al. loc. cit.*). At least nine metabolites contributed to the urinary radioactivity, of which 8·3% was accounted for by hippuric acid, 10·9% by desmethyldichlorvos, 27% by dichloroethanol glucuronide and 3·1% by urea. The alternative routes of dichlorvos metabolism are outlined in Fig. 1.

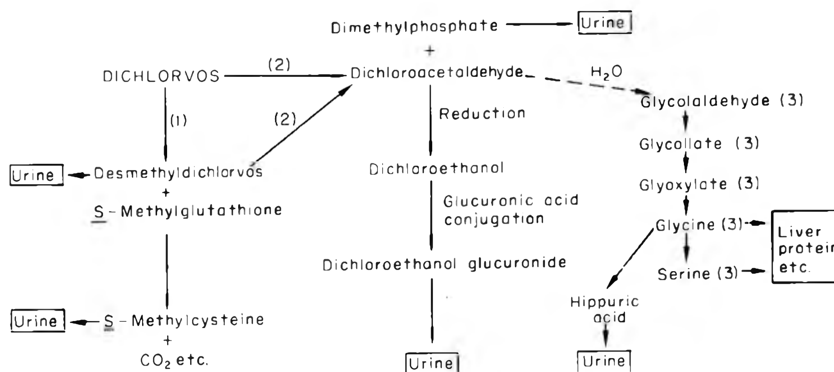

Fig. 1. Dichlorvos metabolism; (1) Glutathione-dependent demethylation and oxidative *O*-demethylation; (2) Hydrolysis by liver and blood enzymes; (3) ^{14}C incorporated into normal body processes.

Hydrolysis was important in all the species studied (Hutson & Hoadley, *loc. cit.*), the dichloroacetaldehyde produced being reduced to dichloroethanol and conjugated or dechlorinated, with subsequent utilization of the two-carbon fragments in intermediary metabolism. Demethylation was generally of less importance, except in the mouse, in which desmethyldichlorvos accounted for at least 18·5% of the administered radioactivity. The metabolic picture obtained in the rat with inhaled dichlorvos (Hutson *et al. loc. cit.*) was similar to that after oral dosage.

Short- and long-term oral and inhalation studies in pigs, using ^{32}P-, ^{36}Cl- and ^{14}C-labelled material, showed that detoxication, while again rapid, proceeded almost entirely by hydrolysis of the phosphate–vinyl bond and subsequent dechlorination (Page *et al. Arch. Tox.* 1972, **30**, 19). Intact dichlorvos rapidly disappeared from the blood, and no tissue accumulation of organochlorine residues was detected (Potter *et al. J. agric. Fd Chem.* 1973, **21**, 163). In the studies with ^{36}Cl-labelled dichlorvos, the radioactivity retained in the tissues was identified as chloride ion (Page *et al. Toxic. appl. Pharmac.* 1971, **19**, 378). The apparent insignificance of the dealkylation pathway in this species was unexpected, since several *in vitro* studies on pig liver and lung tissue had demonstrated the presence of enzymes capable of demethylating dichlorvos by either glutathione-dependent or oxidative pathways. It is possible that demethylation becomes more important with higher doses of dichlorvos. Page *et al.* (1972, *loc. cit.*) reported that the formation of small amounts of desmethyldichlorvos was easily demonstrated in pig blood containing 1 or 0·1 ppm dichlorvos, but not with a concentration of 0·01 ppm. The same effect was found with human blood. In blood from both species, the major product was dimethylphosphoric acid.

Conclusion

It thus appears that the rapid metabolism and deactivation of dichlorvos is an achievement shared by a variety of animal species, including man. This is obviously a factor of major importance with respect to any pesticide, since these are essentially materials of high biological activity. It is equally important to establish that man will not be exposed to levels of the pesticide that could overload the available metabolic capacity, particularly in individuals in whom this might be impaired for physiological or clinical reasons. In this respect, too, the evidence available on dichlorvos appears to be generally favourable. Gillett *et al.* (*loc. cit.*) concluded from an exhaustive survey, particularly of the data on resin strips, that while some aspects needed further study, the available information offered no substantial evidence of hazard to man from the use of dichlorvos in the prescribed manner.

[A. M. S.—BIBRA]

THE GREAT POTATO DEBATE—END OF THE STORY?

A heavy attack has been launched in recent months against the hypothesis that consumption of blighted potatoes by women during pregnancy is a critical aetiological factor in cases of anencephaly and spina bifida in the Western world (*Cited in F.C.T.* 1973, **11**, 311). Widespread epidemiological studies have provided little support for the theory, and tend rather to confirm the doubts expressed in our last review on this subject (*ibid* 1973, **11**, 1134).

In part, the theory stemmed from an observed correlation between the anencephalic stillbirth rate in Scotland and the severity of potato blight in the west of the country during each preceding spring over the period 1946–1955. Stressing this point, Kinlen & Hewitt (*Br. J. prev. soc. Med.* 1973, **27**, 208) have recently commented on the improbability that many of the malformed foetuses included in the analysis could have been exposed to any constituents of potatoes grown in the west of Scotland, since this area provides only a small proportion of the total Scottish crop. On the assumption that embryonic damage occurred in early pregnancy, these authors compared national and regional data on potato blight in Scotland with the quarterly incidence rates of anencephaly, but no temporal correlation was found. This finding was in keeping with the recent analysis of data relating to the Edinburgh area (*Cited in F.C.T.* 1973, **11**, 1135).

The original suggestion that potato consumption might be responsible in the UK for 95% of the cases of anencephaly and spina bifida (together designated ASB for convenience) also linked a high seasonal peak of these defects in South Wales with the quality of potatoes during the previous year. It was suggested that in coal-mining areas many individuals kept their harvested home-grown potatoes indoors, in conditions ideal for rapid deterioration. More recently, however, a retrospective survey of 240 infants born with neural-tube malformations in Glamorgan between 1964 and 1966 has failed to reveal any association between this fact and the consumption and storage of home-grown potatoes by the families concerned (Roberts *et al. Br. J. prev. soc. Med.* 1973, **27**, 214). It was found that 61% of the malformed infants and 60% of a group of matched controls were not exposed to homegrown potatoes, and among the remaining families there was no evidence of longer storage of potatoes by the affected group.

In another recent retrospective survey (Clarke *et al. Br. med. J.* 1973, **3**, 251), comparisons were made between the mothers of 83 children with spina bifida (mostly schoolchildren with meningomyelocele) and those of 85 control children carefully matched with respect to sex, age and social background. The mean consumption of potatoes by the mothers in the experimental group (1·63 kg/week) was not considered to differ significantly from that by the control mothers (1·8 kg/week). Of the seven other comparisons associated with potatoes, only one—the tendency to frequent chip shops—showed a significant difference, the mothers of children with spina bifida being more likely to obtain potatoes from this source. While chip shops tend to use the cheapest potatoes available, the frequent purchase of potatoes from such a source is likely to be more significant as an indication of poor dietary habits, possibly associated with indifferent maternal health.

In a previous issue (*Cited in F.C.T.* 1973, **11**, 1134), we referred to several recent surveys, in the USA, Canada and Chile, which have failed to link the incidence of ASB with exposure to blighted potatoes. Another area of particular interest is Ireland, where the *per capita* consumption of potatoes is high and where infection of the annual crop by the blight fungus *Phytophthora infestans* is prevalent. Elwood & MacKenzie (*Nature, Lond.* 1973, **243**, 476) have now presented the results of a multivariate analysis comparing secular trends in the annual incidence rates of neurological malformations in a Dublin maternity unit with data on potato-blight outbreaks in the Irish Republic during this century. No linear correlation was found between the incidence of ASB and the number of blight outbreaks during the previous year. Slightly further north, a community study carried out in Belfast over a 4-year period (1964–1968) by Elwood & Nevin (*Br. J. prev. soc. Med.* 1973, **27**, 73) demonstrated a high incidence of neural-tube defects (anencephalus 4·2 and spina bifida 4·5/1000 total births), with a markedly higher proportion occurring in the lower

social groups. It revealed no correlation, however, with overall potato consumption or potato blight or with the consumption of nitrite-cured cooked meats, which have also been tentatively suggested as showing some link with the incidence of anencephaly (*Cited in F.C.T.* 1973, **11**, 1164).

Further defence of the potato has come from Field & Kerr (*Lancet* 1973, **ii**, 507) as a result of a survey in Australia, where potato consumption is considerably less than in most western European countries. No annual relationship was evident between ASB incidence and indices of potato consumption or potato blight in New South Wales over the years 1950–1972. Since late blight affects autumn potatoes (marketed in June and July), an increased incidence of neural-tube defects would, according to the original theory, be anticipated early in the following year. No hint of any such increase was noted in this study.

In replying to his critics, Renwick (*ibid* 1973, **ii**, 562) did not consider that the Australian data convincingly refuted his hypothesis as applied to the UK, since the severity of blight, annual potato consumption and annual ASB rates differ significantly in the two countries. Renwick stressed that his theory was that 95% of ASB occurrences in the UK could be prevented by avoidance of the potato during early pregnancy. Some convincing evidence against this hypothesis would appear to be that collected by Dr. N. C. Nevin of Belfast, whose preliminary findings in a potato avoidance trial carried out on 87 pregnant women who had given birth to a congenitally deformed baby were reported recently in the daily press (Cunningham, *Guardian* 17 January 1974). Of these 87 subjects, 27 avoided potatoes during a subsequent pregnancy, while the remainder continued on their normal diet. Among the 47 women from the control group whose deliveries have so far been reported, three have given birth to babies with ASB. This compared with two similar abnormalities among the 16 deliveries reported in the group on the potato-free diet. Although the numbers involved are too small to allow any statistical analysis, these findings provide no support for the potato hypothesis and their compiler has also pointed to the fact that, while the consumption of potatoes in Ulster has dropped by almost a third over the last 5 years, there has been no decrease in the proportion of malformed babies.

When rats and marmosets were fed blighted-potato concentrate during pregnancy, no abnormalities were observed in foetal rats, but cranial osseous defects were found in four out of 11 foetal marmosets (*Cited in F.C.T.* 1973, **11**, 313). More recent feeding trials conducted by the same workers (Poswillo *et al. Teratology* 1973, **8**, 339) have been aimed at isolating the factor responsible for central nervous system abnormalities found in the marmoset. Concentrates of normal potatoes, of rejected tubers and of potatoes infected with *Erwinia carotovora* were fed to pregnant marmosets under similar conditions. No gross abnormalities were identified, but behavioural defects were noted in 50% of the animals whose mothers had ingested "rejected" concentrate.

Terpenoid derivatives such as rishitin and phytuberin (*Cited in F.C.T.* 1973, **11**, 312) and steroid glycoalkaloids including solanine (*ibid* 1973, **11**, 311) have been implicated as possible aetiological factors in ASB. Since high levels of glycoalkaloids were reported by Poswillo *et al.* (*loc cit.*) in both the rejected and the blighted concentrates, it is unlikely that these high levels were significant in relation to the cranial defects observed in the earlier experiment. Terpenoid derivatives, although present in high concentrations in the blighted concentrate, did not appear to be associated with abnormalities. Swinyard & Chaube (*Teratology* 1973, **8**, 349) failed to produce neural-tube defects in the offspring of rats given daily ip injections of solanine or total potato glycoalkaloids in doses of 5–10 mg/kg for various periods during pregnancy. A relatively high incidence of hydronephrosis, hydrour-

eter and mild rib malformation was recorded, however. A similar lack of neural-tube malformations followed administration of blighted potato to the rats by gavage, and no teratogenic effects were associated with glycoalkaloid treatment of rabbits or chick embryos.

Shepard (*Lancet* 1973, **i,** 96) has suggested that cytochalasin, which has been shown to produce neural-tube malformations in chick embryos (*Cited in F.C.T.* 1973, **11,** 312), may be present in blighted potatoes. When chloroform extracts of potato samples used in the experiments of Poswillo *et al.* (*loc. cit.*) were examined for this activity, cytochalasin could only be detected in the original blighted concentrate. Even here the actual level of activity was low, and it was estimated that the total daily dose given to each marmoset in the original trial was only about 3 μg cytochalasin B. Involvement of organic mercury in the production of cranial defects was considered unlikely, since the highest level of mercury in the original blighted concentrate (0·004 mg/kg fresh weight) was just within the normal range for potatoes.

The only real clue as to the origin of ASB defects lies in the fact that some incidence of these deformities has been found in every human community in which they have been sought (Stevenson *et al. Bull. Wld Hlth Org.* 1966, **34,** Suppl. p. 1, cited from Emanuel & Sever, *Teratology* 1973, **8,** 325). The fact that skeletal malformations characteristic of ASB have been recorded, for example, in ancient Egypt (Emanuel & Sever, *loc. cit.*) and modern Europe alike must raise doubts as to the feasibility of a single food-associated teratogen being responsible for these congenital defects. Dietary constitution varies widely according to both custom and geographical location, and so any truly ubiquitous disease is unlikely to be attributable to a single environmental factor. This argument has led Emanuel & Sever (*loc. cit.*) to propose a hypothesis for the aetiology of ASB, involving an interaction of genetic and environmental factors. Three categories of causal factors have been implicated by these authors, namely the genetic, the immediate—pertaining to the pregnancy in question—and the intergenerational—pertaining to the growth and development of the mothers of ASB children.

A genetic aetiology has long been considered likely, in view of the well-established 4–5% risk of recurrence following the birth of the first affected child in a sibship (Nance, *Nature, Lond.* 1969, **224,** 373). Nevertheless, such a hypothesis is hard to reconcile with the report of Yen & MacMahon (*Lancet* 1968, **ii,** 623) that not one of 108 twin pairs taken from their studies and those of other workers were concordant with respect to the incidence of either spina bifida or anencephaly. As Nance (*loc. cit.*) has pointed out, however, monozygotic twins can also differ in their cytoplasmic endowment, and so ASB may still be inherited by way of cytoplasmic abnormality.

The evidence in favour of an intergenerational aetiology is again speculative, and consists largely of hypothetical correlations between socio-economic factors and the incidence of ASB in a given population. Thus, it may be more than coincidental that high unemployment and migration rates have preceded the two major epidemic waves of ASB in the UK during this century (Emanuel & Sever, *loc. cit.*). It is also of interest here that the epidemiological survey of Clarke *et al.* (*loc. cit.*), mentioned earlier, lent some support to the view that poor dietary habits, possibly associated with maternal ill-health, might be implicated in the aetiology of spina bifida.

Several studies in the UK and the USA have pointed to an inverse relationship between the hardness of local water supplies and the incidence of ASB (Crawford *et al. Lancet* 1972, **i,** 988). Here again, rather than suggesting that calcium deficiency is directly responsible

for ASB conditions. these data are much more likely to reflect a low state of health of mothers suffering. perhaps. from mineral depletion.

At present, therefore, no single teratogen can be implicated in the aetiology of ASB defects, and a more tenable hypothesis may involve a complex interplay of biological and environmental factors.

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Fd Cosmet. Toxicol. Vol. 12 pp. 777–801. Pergamon Press 1974. Printed in Great Britain

TOXICOLOGY: BIBRA ABSTRACTS AND COMMENTS

COLOURING MATTERS

2747. Feeding studies on auramine

Zeller, H., Birnstiel, H., Freisberg, K. O., Kirsch, P. u. Hempel, K. H. (1973). Zur Frage der chronischen Toxizität von Auramin. *Naturwissenschaften* **60,** 523.

Auramine has been suspected of having some carcinogenic potential, and its use as a food colouring has been condemned by WHO (*Cited in F.C.T.* 1972, **10,** 257). A series of feeding experiments in rats has been described in the paper cited above. Technical auramine was fed in the diet for a period of 28 days at levels of 100 and 400 ppm, for 90 days at 50, 100 and 200 ppm (with observation in some animals for 2 yr), and for 2 yr at 50, 100 and 200 ppm.

In the 28-day feeding trial, 400 ppm auramine caused, in both sexes, a significant reduction in food consumption and body weight, with an accompanying reduction in the absolute and relative liver and kidney weights. The lower dose had no significant effect. With the two higher doses used in the 90-day trial, the liver weight expressed as a proportion of body weight increased in males, as did the liver-/heart-weight ratio in the 50 ppm group. No lesions or tumours attributable to treatment were found in any of the organs examined macroscopically or histologically after the 90-day feeding trial, and in animals kept for 2 yr, either for observation or on the test diet, there was no significant increase in mortality compared with controls. Clinical parameters and histological changes in the treated animals did not differ from those of the controls and none of the treatments had any effect on the incidence or time of development of tumours. The incidence of liver-cell adenomas could not be correlated with dosage.

There was thus no evidence in these trials to support the contention that auramine has any tumorigenic effect.

FLAVOURINGS, SOLVENTS AND SWEETENERS

2748. Metabolism of isopropanol in rats

Nordmann, R., Ribiere, C., Rouach, H., Beauge, F., Giudicelli, Y. & Nordmann, J. (1973). Metabolic pathways involved in the oxidation of isopropanol into acetone by the intact rat. *Life Sci.* **13,** 919.

The widely-used solvent, isopropanol, is known to be oxidized to acetone in man and experimental animals (*Cited in F.C.T.* 1970, **8,** 224), but the exact pattern of metabolism has been the subject of some controversy in the past. Although the non-specific hepatic enzyme, alcohol dehydrogenase (ADH), catalyses the oxidation of methanol, ethanol and isopropanol, the elimination kinetics of isopropanol are quite different from those of the two lower homologues (*ibid* 1970, **8,** 224). The mechanism of isopropanol metabolism is also of interest since, of the first nine aliphatic alcohols administered acutely to rats in large dosage, isopropanol and ethanol were alone in inducing fatty liver.

In the acute study cited above, isopropanol administered orally to rats in a dose of 6 g/kg body weight induced a marked increase in the blood concentration of isopropanol, which attained its maximum level (600 mg/100 ml) within 8 hr. Blood-acetone levels rose steadily to maximum values in the region of 150 mg/100 ml after 20 hr. When a dose of 1 g isopropanol/kg was administered ip, the early high levels of the alcohol in the blood declined linearly (at a rate of 17 mg/100 ml blood/hr) over a 6-hr period, by the end of which the blood-acetone levels had increased slowly to a level of about 70 mg/100 ml.

When pyrazole, a potent inhibitor of ADH and to a lesser extent of catalase, was administered by gavage 23 hr before the isopropanol (1 g/kg) injection, the clearance of isopropanol from the blood was reduced and the rate of acetone production was markedly delayed. In contrast, 3-amino-1,2,4-triazole, which inhibits catalase but not ADH, had no significant effect on isopropanol clearance and acetone accumulation in the blood, when given by ip injection 1 hr before the isopropanol administration.

The effect of isopropanol on the hepatic redox state was investigated by determining the hepatic lactate/pyruvate and β-hydroxybutyrate/acetoacetate ratios 1·5 hr after oral administration of 6 g isopropanol/kg or an ip injection of 1 g/kg. Of the two ratios examined, only the β-hydroxybutyrate/acetoacetate ratio was affected by isopropanol, given orally or ip, and the increase was only slight.

The authors conclude from these findings that catalase does not participate significantly in the oxidation of isopropanol, at least in the rat. Although the evidence points to a major role for ADH in isopropanol metabolism, isopropanol—unlike ethanol—failed to modify the liver extramitochondrial NAD^+/NADH ratio (as judged by the hepatic lactate/pyruvate ratio). This may be attributed to the further metabolism of acetone by the rat liver. A slight decrease in the intramitochondrial NAD^+/NADH ratio (as reflected in the effect on the β-hydroxybutyrate/acetoacetate ratio) can be added to previous findings of an isopropanol-induced reduction in the respiratory quotient, to suggest the possibility of a direct effect of isopropanol on the redox state of the rat mitochondria.

[These findings are in keeping with our previous report of differences in the elimination kinetics of ethanol and isopropanol (*Cited in F.C.T.* 1970, **8**, 224), but the aetiology of isopropanol-induced fatty liver remains obscure.]

2749. Aspartame metabolism studies

Oppermann, J. A., Muldoon, E. & Ranney, R. E. (1973). Metabolism of aspartame in monkeys. *J. Nutr.* **103**, 1454.

Oppermann, J. A., Muldoon, E. & Ranney, R. E. (1973). Effect of aspartame on phenylalanine metabolism in the monkey. *J. Nutr.* **103**, 1460.

Aspartame (L-aspartyl-L-phenylalanine methyl ester), the sweetness of which is some 180 times that of sugar, has been suggested as a possible low-calorie sweetener for foodstuffs.

Aspartame is hydrolysed in the gut to methanol, aspartate and phenylalanine, and the first paper cited above reports investigations into the metabolic behaviour of aspartame compared with that of its metabolic products. Young female rhesus monkeys were given aspartame labelled with ^{14}C in the methyl, aspartyl or phenylalanine moieties, and the amounts of radioactivity excreted in expired air, urine and faeces and incorporated into plasma proteins were measured and compared with the metabolic picture following administration of the three individual components, [^{14}C]methanol, [^{14}C]aspartic acid

and [^{14}C]phenylalanine. The labelled compounds were each administered by oral intubation in a single dose of 0·068 mmols/kg (10–50 μCi/dose). Apart from a slight delay attributed to the transfer of aspartame into the small intestine and its hydrolysis there, the pattern of CO_2 production and of urinary and faecal elimination was the same for the parent compound and its components. The phenylalanine moiety of aspartame was largely incorporated into body protein, only some 20–25% being excreted.

In the second half of the experiment (second paper cited above), rhesus monkeys were pretreated with 15 or 60 mg aspartame/kg/day for 10 days and were then given an iv infusion of [U-^{14}C]phenylalanine. Pretreatment with aspartame had no significant effect on the time of disappearance of [^{14}C]phenylalanine from the plasma or on the rate of conversion of this amino acid to tyrosine and to CO_2.

It may be concluded, therefore, that aspartame is broken down first to normal dietary constituents and is thus incorporated into established metabolic pathways, and it is also apparent that repeated aspartame ingestion does not materially modify phenylalanine metabolism in the rhesus monkey.

ANTIOXIDANTS

2750. BHA as a carcinogenesis inhibitor

Wattenberg, L. W. (1973). Inhibition of chemical carcinogen-induced pulmonary neoplasia by butylated hydroxyanisole. *J. natn. Cancer Inst.* **50,** 1541.

In a previous study carried out by the author named above (*Cited in F.C.T.* 1973, **11**, 328), butylated hydroxyanisole (BHA) fed at a dietary level of 10,000 ppm to mice was shown to reduce the induction of forestomach tumours by benzo[*a*]pyrene (BP) or 7,12-dimethylbenz[*a*]anthracene (DMBA) also administered in the diet. Further experiments are now reported in which BHA appears to be able to inhibit chemical carcinogenesis at sites remote from the route of administration of the carcinogen.

In the first part of the study, groups of mice fed BHA at a level of 0·5% in the diet were given BP (3 mg) or DMBA (0·75 mg) by oral intubation on days 4 and 18 of the BHA-feeding period, urethane (10 mg) as a single ip dose on day 13, or uracil mustard orally in doses of 0·05 mg on days 4, 7, 10 and 14. The mice were transferred to a BHA-free diet after 2–3 wk and were killed about 20 wk after the start of the BHA feeding. In the second part of the experiment, BHA (1%) and/or a carcinogen were added to the diet, the levels used being 0·05% dibenz[*a,h*]anthracene (DBA), 0·005% DMBA and 0·005% 7-hydroxy-DMBA.

In every instance in both studies the incidence of pulmonary adenomas was reduced by concurrent administration of BHA in the feed compared with that in mice similarly treated with the carcinogens but kept on a BHA-free diet. The mechanism by which BHA inhibits carcinogenesis at a remote site is not understood. It seems likely that it reacts either with the ultimate carcinogenic metabolite in the lung or with a close metabolic precursor.

AGRICULTURAL CHEMICALS

2751. Cooking and ethylene thiourea

Newsome, W. H. & Laver, G. W. (1973). Effect of boiling on the formation of ethylenethiourea in zineb-treated foods. *Bull. env. contam. & Toxicol. (U.S.)* **10,** 151.

Ethylene thiourea (ETU) is a breakdown product of the ethylenebis(dithiocarbamate) fungicides, and has recently been shown in experimental animals to have teratogenic properties (*Cited in F.C.T.* 1974, **12**, 282) as well as a capacity for exerting carcinogenic and other toxic effects on the thyroid (*ibid* 1973, **11**, 702). There has already been a report of ETU residues in apples (Newsome. *J. agric. Fd Chem.* 1972, **20**, 967) and it has now been shown that such residues may increase during cooking.

Carrots and spinach were sprayed with a suspension (1 mg/ml) of a commercial zineb preparation, containing 82·9% zinc ethylenebis(dithiocarbamate), allowed to dry naturally and then boiled in water under reflux for 15 min. At the end of this time, ETU was present in the cooked carrots and spinach at levels of 0·248 and 0·880 ppm, respectively, compared with levels of 0·073 and 0·182 ppm in the uncooked samples, while the cooking water from the carrots and spinach contained 0·838 and 2·70 ppm ETU respectively. When apples and tomato samples containing 50 ppm added zineb were similarly treated, ETU levels increased from 0·033 and 0·067 ppm in the raw samples to 0·300 and 0·311 ppm after cooking. When 5 g carrots with 2·50 μg zineb were refluxed in 15 ml water for various times, the level of ETU formed was found to increase linearly with time, reaching about 0·8 ppm after 60 min from an initial level of the order of 0·05 ppm.

[Although a clear increase in the levels of ethylene thiourea in foods was recorded during normal cooking periods, the levels found were still below 1 ppm, despite the fact that zineb had been applied immediately beforehand. The finding must thus be kept in perspective, since residues of the parent fungicide resulting from normal agricultural practice would be expected to be much lower than the levels of zineb used in this study.]

2752. The maze of BHC metabolism

Karapally, J. C., Saha, J. G. & Lee, Y. W. (1973). Metabolism of lindane-^{14}C in the rabbit: Ether-soluble urinary metabolites. *J. agric. Fd Chem.* **21**, 811.

The metabolism of lindane (γ-BHC) in rats has been shown to be more complex, judging from the numbers of urinary metabolites, than was previously thought (*Cited in F.C.T.* 1973, **11**, 691). The same observation applies to mammalian metabolism of BHC in general, and the paper cited above presents data on the metabolites produced in rabbits fed ^{14}C-labelled lindane in gelatin capsules over 26 wk.

Urinary and faecal excretion accounted, respectively, for 54 and 13% of the administered radioactivity by the end of the dosing period. The ether-soluble fraction of urinary metabolites amounted to 56% of the total in the urine. By infra-red spectroscopy, the authors were able to identify 2,3,5-, 2,4,5- and 2,4,6-trichlorophenols and 2,3,4,6-tetrachlorophenol. Three more compounds were positively identified by gas chromatography and mass spectrometry, namely 2,3- and 2,4-dichlorophenols and 2,3,4,5-tetrachlorophenol. Tentative identification by gas chromatography was achieved in the case of the 2,5-, 2,6- and 3,4-dichlorophenols, 2,3,4-, 2,3,6- and 3,4,5-trichlorophenols, pentachlorophenol, 1,2-dichlorobenzene, 1,2,4-trichlorobenzene, 1,2,3,4- and 1,2,4,5- and/or 1,2,3,5-tetrachlorobenzenes, and pentachlorobenzene.

The most abundant of these metabolites was 2,4,5-trichlorophenol, followed in decreasing order by 2,3,5- and 2,4,6-trichlorophenol, 2,3,4,6-tetrachlorophenol, 2,3- and 2,4-dichlorophenols and 2,3,4-trichlorophenol, the remaining metabolites being present in very small or trace amounts.

These findings confirm the complexity of the processes involved in lindane metabolism

in mammals. There is no adequate evidence for many of the metabolic pathways which must be concerned in the formation of these metabolites, apart from the evident processes of dehydrochlorination, dehalogenation, dehydrogenation and hydroxylation.

2753. More on paraquat toxicity

Fisher, H. K., Clements, J. A. & Wright, R. R. (1973). Pulmonary effects of the herbicide paraquat studied 3 days after injection in rats. *J. appl. Physiol.* **35,** 268.

Smalley, H. E. (1973). Toxicity and hazard of the herbicide, paraquat, in turkeys. *Poult. Sci.* **52,** 1625.

Since our last broad comment on paraquat toxicity (*Cited in F.C.T.* 1972, **10,** 700), experiments in various animal species have continued to throw more light on the nature of the lung pathology induced by paraquat and the mechanism whereby it is achieved. Attention has already been drawn to the changes in surfactant in animal lungs (*ibid* 1972, **10,** 704) and this is considered again in the first paper cited above. Rats were given a tail-vein injection of paraquat dichloride (27 mg/kg) and their lungs were examined 3 days later. Of the 129 rats treated, 24 (19%) died within this period. The survivors were apathetic, moved only when alarmed, and breathed rapidly, shallowly and with intermittent gasping. Emaciation, cyanosis and red-brown crusting of nares, eyelid and nailbeds were seen. Body weight was reduced by 17%, but the lungs were more than twice their control weight because of an increase in fluid content and accumulation of blood solids. The maximum ventilatory capacity of the affected lungs was diminished, and alveolar surface forces were increased. Lung washings from these rats produced no measurable surfactant, whereas washings from the lungs of control rats yielded amounts of surfactant equivalent to 5–6 mg/kg. Of the lipids measured in whole lungs, only anenoic lecithins showed a reduction after paraquat treatment. The severe atelectasis induced by paraquat was therefore probably attributable to increased alveolar surface forces, to which lung oedema may have been a contributory factor.

The second paper cited reports that a single oral dose of 290 mg paraquat/kg was consistently lethal to turkey hens; toxic effects followed doses between 30 and 200 mg/kg, the no-effect dose being 30 mg/kg. Within the LD_{50} range (250–280 mg/kg), paraquat was lethal within 1–5 days, producing diarrhoea within 4 hr and later anorexia, dullness of eyes, drooping of wings, ruffling of feathers and extreme weakness. Lethal doses produced a brief but violent terminal convulsion. Survival involved no toxic sequelae. Necropsy showed severe gastro-enteritis affecting the tract from the proventriculus to the small intestine. The heart, serous intestinal coat, vertebral column and voluntary muscles showed petechial haemorrhages.

Direct application of paraquat to the skin of turkeys induced blisters and transparency of the skin. The lethal dose by this route was 400–500 mg/kg. Injections of 100 mg paraquat/kg given ip were uniformly lethal within 1 day, causing almost immediate incoordination followed by cyanosis. Intravenous injections of 20 mg paraquat/kg or more were promptly lethal. Birds dying within 1 day of an ip or iv injection of paraquat showed engorged viscera. Those dying after several days had lung congestion, kidney congestion and blanched and friable liver. Turkeys allowed to range on Bermuda grass which had been sprayed 24 hr previously with 100 times the recommended rate of paraquat application developed no toxic signs.

2754. Parathion and testosterone metabolism

Stevens, J. T. (1973). The effect of parathion on the metabolism of ^{3}H-testosterone by hepatic microsomal enzymes from the male mouse. *Pharmacology* **10**, 220.

In a recent issue (*Cited in F.C.T.* 1974, **12**, 572) we suggested that commonly used pesticides should be examined with respect to possible effects on the male reproductive system. *In vitro* studies have already laid the foundations for such work by showing, for example, that various organophosphates can alter the amount of polar metabolite formed from testosterone by rat microsomal preparations. The present paper elaborates a simplified method for the *in vitro* detection and estimation of individual testosterone metabolites and highlights the effect of parathion on the formation of these metabolites as a specific example.

Male Swiss-Webster mice were killed and liver microsomes were separated by differential centrifugation. After incubation with [4-^{3}H]testosterone, the microsomal preparation was analysed for metabolites of the steroid hormone by thin-layer chromatography and radioactivity determinations. The main polar metabolites of testosterone formed by mouse-liver microsomes were found to be 16α- and 6β-hydroxytestosterone. The other polar metabolite was 7α-hydroxytestosterone, and three non-polar metabolites, androstan-β,β-diol, dihydrotestosterone and androstandione, were also identified. Addition of parathion (0·1 mM) to the incubation mixture reduced the formation of the two major metabolites (by 40·5 and 61·4% for the 16α- and 6β-hydroxy metabolites, respectively) but had no significant effect on the other metabolites. While 0·01 mM-parathion did not affect the overall metabolic pattern, there were indications of some inhibition of testosterone metabolism at this as well as the higher level of parathion.

[Further work on the potential inhibitory effects of insecticides on testosterone metabolism could usefully be combined with an *in vivo* investigation of androgen accumulation by the prostate gland, as reported earlier for 2,4,5-T (*Cited in F.C.T.* 1974, **12**, 572).

2755. More effects of parathion

Kibler, W. B. (1973). Skeletal muscle necrosis secondary to parathion. *Toxic. appl. Pharmac.* **25**, 117.

Talens, G. & Woolley, D. (1973). Effects of parathion administration during gestation in the rat on development of the young. *Proc. West. pharmac. Soc.* **16**, 141.

The first paper cited above describes the development of skeletal muscle necrosis in rats given daily ip injections of parathion in doses of 1 or 1·3 mg/kg for 2 wk. The lesions were attributed to excessive liberation of acetylcholine. The acute effects of each injection (tremor, respiratory embarrassment, cyanosis, lethargy and a depressed level of consciousness) became less marked after 10 days, by which time no residual acute symptoms persisted between injections. Skeletal muscle weakness became apparent between days 5 and 9, and progressive myopathy reached a peak on day 8 in animals given the dose level of 1 mg/kg/day. Animals given 1·3 mg parathion/kg/day showed muscle-fibre degeneration by day 3. Adaptation occurred after a few days, preventing further progression of the myopathy. The histopathology of the muscle lesions induced by parathion resembled that reported after repeated doses of paraoxon.

Parathion has been reported to be only feebly teratogenic in rats, but definitely teratogenic in the chick and quail (*Cited in F.C.T.* 1972, **10**, 411), the most sensitive index of toxicity during pregnancy probably being a reduction in foetal weight. The second paper cited

above reports a study in which pregnant rats were given sc injections of 1·5 or 2 mg/kg daily for 4 days from day 1, 7 or 13 of gestation. Toxic symptoms in the dams were more severe during treatment in the third trimester, when several animals given the higher dose died. The only two stillborn pups were born to rats injected during the later part of gestation. Pup deaths among treated animals totalled nine (including one litter of six) in wk 1 and four in wk 2, compared with two in the fortnight in the control group. The average litter size was 11, without difference between the groups, suggesting an absence of foetal resorptions. Although growth was less rapid in treated pups than in controls during wk 1, body weight was normal by the end of wk 2. The development of the startle reflex, eye opening and the righting reflex was delayed in pups from treated dams. Slower body growth and reflex development may have been due to maternal factors affecting the early life of the pups, since maternal behaviour of the treated dams was poor and the treatment may have impaired lactation. A direct effect on the foetus could not be excluded, however. Acetylcholinesterase activity of the brain was found to be normal at birth, although a related study indicated that, at that time, this activity was depressed in the dams, particularly in those treated during the third trimester.

FEED ADDITIVES

2756. Arsanilic acid and the pig

Ledet, A. E., Duncan, J. R., Buck, W. B. & Ramsey, F. K. (1973). Clinical, toxicological, and pathological aspects of arsanilic acid poisoning in swine. *Clin. Toxicol.* **6**, 439.

Pigs given doses of arsanilic acid in excess of the recommended feed addition of about 100 ppm have been found to develop tremor and incoordination of the limbs (*Cited in F.C.T.* 1969, **7**, 390). The effects of feeding some ten times the recommended quantity have now been described in detail. Pigs were fed 1000 ppm arsanilic acid in a balanced ration continuously for up to 27 days and were killed in groups of three at 2- or 3-day intervals from day 4, for autopsy and assay of tissue samples for arsenic (As).

The highest As levels were found in the kidney and liver, with means of 8 and 6 ppm, respectively, over the whole period. In these two tissues and in the skeletal muscle, ribs and blood, maximal As levels were reached on or before day 13 of feeding. Arsenic accumulation did not reach a maximum in the peripheral nerves and other parts of the nervous system until days 16–21, however. Some pigs developed roughening of the coat on day 3 and had mild diarrhoea within 24–36 hr of this. After 2–3 days, diarrhoea gave way to constipation, which then subsided spontaneously. Cutaneous hyperaemia and hyperaesthesia were evident in some pigs by day 5. Appetite was not affected. Incoordination sometimes appeared by day 6, progressing by day 15 to posterior paresis and sometimes by day 18 to quadriplegia. Autopsy revealed no gross lesions attributable to arsanilic acid toxicity, but there were consistent histopathological changes, involving demyelination of peripheral nerves, optic nerves and optic tracts.

In a second phase of the experiment, in which pigs were fed 1000 ppm arsanilic acid for 19 days, the animals were killed and autopsied 3, 6 or 11 days after withdrawal of the drug from the diet. Clearance of As from edible tissues was found to be rapid, skeletal muscle retaining less than 0·3 ppm As by day 3 (from a level of 0·9 ppm at termination of arsanilic acid administration). In a third phase, six pigs which developed posterior paresis while maintained on the 1000 ppm diet showed no clinical improvement when transferred to an

As-free diet for 10–38 days before being killed. Four of the six remained unchanged, while two showed further deterioration.

PROCESSING AND PACKAGING CONTAMINANTS

2757. Effect of adipates on foetal development in the rat

Singh, A. R., Lawrence, W. H. & Autian, J. (1973). Embryonic-fetal toxicity and teratogenic effects of adipic acid esters in rats. *J. pharm. Sci.* **62,** 1596.

This paper describes the embryonic toxicity and teratogenic effects of seven adipic acid esters given by ip injection to female rats. Six of the esters (with ip LD_{50} values below 6 ml/kg) were given in doses equivalent to 3·3, 10, 20 and 33% of this LD_{50} on days 5, 10 and 15 of gestation. The less toxic di-2-ethylhexyl adipate was administered at dose levels of 1, 5 and 10 ml/kg. Of the four control groups, one was untreated and the others received a 10 ml/kg ip injection of either distilled water, normal saline or cottonseed oil. The animals were killed on day 20 of gestation, 1 day before expected parturition, and the numbers of corpora lutea, resorption sites and viable and dead foetuses were recorded. Both viable and dead foetuses were weighed and examined for gross abnormalities. About 50% were processed for examination of skeletal malformations and the remainder were examined for visceral abnormalities.

Although all the esters affected foetal development to some extent, dicyclohexyl adipate produced the most severe reaction. All four dose levels were associated with a high proportion of resorptions (14·5–20%) and some dead foetuses. In addition there was a 6–10% incidence of gross abnormalities, mainly haemangiomas, twisted legs and compacted head and neck, in the three higher dosage groups. No skeletal abnormalities were found in the groups given the two lower doses of this ester, but one of the 25 foetuses examined at the high dose level had two ribs less than normal and one of 26 given the next highest dose had elongated and fused posterior ribs. No visceral abnormalities were seen in any group.

With dimethyl adipate, a significant number of resorptions occurred only with one of the lower dose levels. No gross, skeletal or visceral abnormalities occurred in the group on the lowest dose (0·0603 ml/kg). The group on the highest dose (0·6028 ml/kg) contained two cases of visceral abnormality (8·3% incidence) and haemangiomas of the neck, shoulder and legs, while five of 26 foetuses had elongated frontal ribs fused to the sternebrae. In the group given 0·3617 ml/kg, one foetus had no tail and four out of 29 had rib malformations. A dose of 0·1809 ml/kg produced one foetus with haemangiomas and two (out of 27) with elongated fused ribs. The highest dose of diisobutyl adipate (1·9833 ml/kg) produced a number of gross and skeletal malformations, but only one visceral abnormality. There were no abnormalities in the lowest dosage group. With diethyl, dipropyl, di-*n*-butyl and di-2-ethylhexyl adipates, there were few gross, skeletal or visceral abnormalities and these occurred mainly with the higher doses. There were two dead foetuses in the group given the highest dose of diethyl adipate and two in the group given the highest and one in that given the second highest dose of di-*n*-butyl adipate. For the most part, foetuses from treated groups weighed less than those from controls, but in most cases the differences were not statistically significant.

The authors conclude that although the adipic acid esters administered did not elicit as great an effect on foetal development as did a series of phthalates in an earlier study (*Cited in F.C.T.* 1972, **10,** 724), the incidence of adverse effects was generally dose-related

and compound-dependent, with the more soluble compounds apparently tending to be more active. The no-effect level established for adipates in this study was approximately 3% of the LD_{50} dose.

MISCELLANEOUS CONTAMINANTS

2758. Heavy metals in cured meat

Kirkpatrick, Diane C. & Coffin, D. E. (1973). Cadmium, lead and mercury content of various cured meats. *J. Sci. Fd Agric.* **24**, 1595.

Human exposure to physiologically inert heavy metals has been the subject of extensive investigation and public concern in recent years. Lead (Pb), cadmium (Cd) and mercury (Hg) are widely distributed in the environment and can enter the food chain in many ways. While their occurrence in food has been closely followed by many authorities, one aspect of this problem that seems to have received relatively little attention is the effect of food preparation on heavy-metal content. In the study cited above, the levels of Pb, Cd and Hg were determined in various Canadian cured-meat products and were compared with the levels encountered overall in the meats making up the meat portion of 'total diets' studied in Canada.

Examination of 190 samples of cured meats obtained from five widely scattered centres in Canada revealed Cd levels ranging from <0·01 to 0·22 ppm, with a mean value of 0·02 ppm. The corresponding range for Pb was <0·01–0·42, with a mean of 0·06 ppm. For these metals, the average concentrations in cured meats were similar to those levels found in the meat portion of the total diets in Canada over the period 1970–71. In the case of Hg, the average concentration in cured meats was 0·006 ppm (range <0·001–0·059), a mean much lower than those (0·051 and 0·028 ppm) found in the meat portion of the Canadian total diets during the same 2 yr. This difference was thought to be due to the fact that fish is included in the meat composite for the total-diet studies.

The authors conclude that the curing process has no significant effect on the levels of Pb, Cd and Hg in meat.

THE CHEMICAL ENVIRONMENT

2759. Elucidating chromium allergy

Shmunes, E., Katz, S. A. & Samitz, M. H. (1973). Chromium-amino acid conjugates as elicitors in chromium-sensitized guinea pigs. *J. invest. Derm.* **60**, 193.

Polak, L. & Frey, J. R. (1973). Studies on contact hypersensitivity to chromium in the guinea pig. Inhibition of the migration of macrophages by chromium salts. *Int. Archs Allergy appl. Immun.* **44**, 51.

Contact hypersensitivity to chromium (Cr) is common both in industry and in the home, and any further light on the mechanism whereby hypersensitivity to different Cr compounds is conferred and on methods for the clinical detection of such hypersensitivity is to be welcomed (*Cited in F.C.T.* 1973, **11**, 506).

The role of amino acids is demonstrated by the first paper cited above, which describes the *in vitro* preparation of conjugates of chromic nitrate ($Cr(NO_3)_3$) and of potassium dichromate ($K_2Cr_2O_7$) with 16 individual amino acids (alanine, arginine, aspartic acid, cystine, glycine, histidine, leucine, lysine, methionine, norvaline, ornithine, phenylalanine, proline,

serine, tryptophan and tyrosine) and their testing by intradermal injection into guinea-pigs. Although the animals were previously sensitized with Cr^{VI} and responded more strongly to challenge with unconjugated Cr^{VI} than to challenge with unconjugated Cr^{III}, they consistently reacted more strongly to amino acid-Cr^{III} conjugates than to amino acid-Cr^{VI} conjugates. This confirms the previously reported observation that reduction of Cr^{VI} to Cr^{III} precedes *in vivo* conjugation and sensitization. Conjugates of sulphur-containing amino acids with Cr^{III} were 3–5 times more potent antigens than unconjugated Cr^{III}, while conjugation decreased the antigenic potency of Cr^{VI}.

The second paper cited describes the investigation of macrophage-migration inhibition in guinea-pigs sensitized to $K_2Cr_2O_7$, using both peritoneal exudate cells produced in response to an ip injection of paraffin oil and cultures of lymph-node cells. Cr^{II}, Cr^{III} and Cr^{VI} all inhibited the migration of exudate cells from Cr-sensitized animals, but did not affect migration of those from normal animals or from Cr-tolerant ones. Slight inhibition of migration occurred with cells obtained from desensitized animals, which were completely unresponsive to epicutaneous tests with $K_2Cr_2O_7$. Migration inhibitory factor (MIF) was detected in the macrophage-free supernatants from lymph-node cells incubated with Cr^{III} but not in those incubated with Cr^{II} or Cr^{VI}, and lymph-node cells from animals tolerant to $K_2Cr_2O_7$ failed to produce any MIF when incubated with Cr^{III}. The results indicate that the direct or indirect macrophage-migration inhibition test for Cr allergy may be more sensitive than the routine skin test. It is suggested that it might take its place in the clinical investigation of cement eczema.

2760. Lead hampers reproduction in rats

McClain, R. M. & Becker, B. A. (1972). Effects of organolead compounds on rat embryonic and fetal development. *Toxic. appl. Pharmac.* **21**, 265.

Hilderbrand, D. C., Der, R., Griffin, W. T. & Fahim, M. S. (1973). Effect of lead acetate on reproduction. *Am. J. Obstet. Gynec.* **115,** 1058.

Gilani, S. H. (1973). Congenital cardiac anomalies in lead poisoning. *Pathologia Microbiol.* **39,** 85.

The neurotoxicity of organolead compounds is well documented (*Cited in F.C.T.* 1967, **5,** 719; *ibid* 1973, **11,** 1147), but less attention has been paid to their possible effects on the foetus. The first study cited above is concerned with the effects of tetraethyllead (TEL), tetramethyllead (TML) and trimethyllead chloride (TLC) on the rat. TEL and TML in corn oil and TLC in saline were given orally at various dose levels to pregnant rats in three daily doses on days 9–11 or 12–14 of gestation. The total doses given over the 3-day periods were 7·5, 15 and 30 mg TEL/kg, 40, 80, 112 and 160 mg TML/kg and 15, 30 and 38 mg TLC/kg. TLC was also given iv in a single dose of 20, 28, 33 or 40 mg/kg on 1 day between days 8 and 15 of gestation, and to overcome the possible effects of the placental barrier, another group was injected intra-amniotically in a dose of 10, 50 or 100 μg/foetus on day 15.

Toxic reactions in the dams included hyperexcitability and weight loss with the lower doses of each compound, and tremor, aggressiveness, spasticity and paralysis with higher doses, these effects becoming apparent 2 or more days after treatment. For each compound, the highest dose of all proved lethal. No gross malformation was seen in any foetus from treated or control dams in spite of a clear association of growth retardation and delay

in bone ossification with all types of treatment. The observed embryotoxic effects and foetal toxicity appeared to be secondary to the maternal toxicity rather than a direct effect on the foetus. In contrast, direct injection of TLC into the amniotic fluid resulted in a dose-related foetal mortality, which reached 96% at the highest dose level. The iv injection of TLC indicated that the placental transfer of TLC was greatly increased when the maternal blood concentration exceeded the threshold saturation capacity of the erythrocytes; below this level the rate of transfer was very limited.

The second paper cited shows the important adverse effect of inorganic lead on reproductive function in the rat. Oral administration of lead acetate (PbAc) in doses of 5 or 100 μg/day for 30 days resulted in mean whole blood concentrations of 19 and 30 μg Pb/100 ml, respectively, in male rats. With the higher dose, the weight of the prostate increased as a result of hyperplasia, reaching up to twice the control weight, and impotence and reduced sperm motility were observed. Four rats (of 20) with an unusually high blood Pb concentration of 50 μg/100 ml also showed a marked reduction in testicular weight. Histological examination of the affected testes revealed lesions of the seminiferous tubules and arrest of spermatogenesis. In treated females, the whole blood concentrations of Pb were higher than in males, 30 and 53 μg/100 ml, respectively, compared with 19 and 30 μg/100 ml. At the 5 μg dose level, oestrous cycles became irregular, while at the 100 μg level the animals had persistent vaginal oestrus after a period of normal oestrus. The latter group developed ovarian follicular cysts, with production of a reduced number of corpora lutea. Hepatic microsomal enzyme activity was significantly depressed in both sexes, indicating a suppression of the normal hepatic detoxication mechanisms.

The third paper cited describes the appearance of cardiac abnormalities in chick embryos exposed at 48 hr to doses of 5–80 μg PbAc/egg, injected into the albumin. Aortic stenosis was seen in 19% of the whole series of embryos treated in this way and examined when 8 days old, the incidence rising from 10 to 30% with increasing Pb dosage. Aortic-valve defects appeared in 16% and valve defects of the pulmonary artery in 5%, the latter lesions being confined to embryos receiving more than 15 μg PbAc. Abnormalities of the atrioventricular valves occurred in 11% and an abnormally thin ventricular wall in 5%. Many of the chick hearts showed several of these defects simultaneously. In 30 control eggs, injected with distilled water at the appropriate time, the only cardiac defects were a malformed pulmonary valve in one embryo and a defective atrioventricular valve in one other. It can be concluded therefore that under the conditions of this study inorganic Pb seriously interfered with cardiac morphogenesis in the chick.

2761. Aldehydes and the blood pressure

Egle, J. L., Jr., Hudgins, Patricia M. & Lai, F. M. (1973). Cardiovascular effects of intravenous acetaldehyde and propionaldehyde in the anesthetized rat. *Toxic. appl. Pharmac.* **24,** 636.

Inhalation of about 3 μg acetaldehyde/ml or slightly more propionaldehyde for 1 min caused an increase in blood pressure in the anesthetized rat, while in a concentration of 12–25 μg/ml these aldehydes accelerated the heart rate (*Cited in F.C.T.* 1973, **11,** 922).

The study cited above demonstrated the biphasic nature of the effects of these aldehydes. A consistent dose-related rise in blood pressure, slightly reduced by adrenalectomy and more markedly diminished by pretreatment with reserpine or phentolamine, was recorded in anaesthetized rats given acetaldehyde or propionaldehyde in an iv dose of 5–10 mg/kg.

This effect indicated that the pressor effects of either aldehyde resulted primarily from vasoconstriction mediated by noradrenaline release from sympathetic nerve endings in the vascular musculature. In larger doses (20–40 mg/kg) each of these two aldehydes sharply reduced blood pressure and induced some bradycardia. Treatment with atropine reduced the hypotensive and cardio-inhibitory effects of the aldehydes, while vagotomy reversed them, indicating that a vagal mechanism was involved.

2762. Industrial exposure to caprolactam

Ferguson, W. S. & Wheeler, D. D. (1973). Caprolactam vapor exposures. *Am. ind. Hyg. Ass. J.* **34,** 384.

ϵ-Caprolactam injected into experimental animals has produced dyspnoea, convulsions and blood-pressure fluctuations (*Cited in F.C.T.* 1964, **2,** 754) but when ingested it is of low toxicity, and the reported effects of industrial exposure have been confined to irritation and occasional sensitization (*ibid* 1964, **2,** 222).

An examination of the medical histories of employees from one monomer plant and two spinning plants has now revealed no differences in general health between employees exposed to caprolactam vapour and those unexposed. Over an 18-yr period no worker had left or been removed from the plants for health reasons, and only three cases of skin irritation, all resulting from direct contact with caprolactam or its concentrated solution, had been sufficiently serious to require medical attention. When five volunteers were exposed experimentally to various concentrations of the vapour in one of the polymer plants, where conditions of low humidity prevailed, transient nose and throat irritation were experienced by some individuals at all levels above 10 ppm. At 25 ppm there was burning of the nose and throat and coughing, and these effects were severe at 104 ppm, when some eye irritation was also recorded. Extreme irritation (choking) was evident at levels of 400–1200 ppm. Discomfort was generally absent in the polymer plant at concentrations up to about 7 ppm, while in the monomer plant, under conditions of 100% relative humidity, no distress or discomfort was noted by any subject at the maximum concentration of 14 ppm.

On the basis of these findings, the authors recommend that employees should not be exposed continuously to more than 5 ppm caprolactam vapour, to prevent discomfort in unacclimatized workers, but they consider that brief exposures up to 50 ppm should be acceptable. It is also recorded (on the basis of other studies not reported in the paper cited) that caprolactam dust may cause skin irritation at 5 but not at 1 mg/m^3, and sound standards of personal hygiene and convenient washing facilities are considered to be important factors in the prevention of excessive exposure to caprolactam dust.

2763. Diphenylhydantoin and pregnancy

Monson, R. R., Rosenberg, Lynn, Hartz, S. C., Shapiro, S., Heinonen, O. P. & Slone, D. (1973). Diphenylhydantoin and selected congenital malformations. *New Engl. J. Med.* **289,** 1049.

Some months ago (*Cited in F.C.T.* 1974, **12,** 151), we discussed the toxicological implications of anticonvulsant therapy during pregnancy, pointing out that the available evidence suggested a somewhat greater risk of malformations in the offspring following the use of diphenylhydantoin (DPH) than was associated with the administration of other common anticonvulsants. Nevertheless, a lack of firm data precluded any sound conclu-

sion to be drawn, especially in view of the established usefulness of this drug. We have since received the results of an epidemiological study, in which the frequency of congenital abnormalities was compared in four groups of children born to non-epileptic mothers or to epileptic mothers subjected to different therapy regimes. Altogether 50,897 pregnancies were studied.

A malformation rate of 6·1% was recorded in the group of 98 infants exposed during the early months of pregnancy to daily DPH treatment of the mother. A significantly lower rate (2·5%) was recorded for the 50,591 children born to non-epileptic women. The authors also found a 5·1% malformation rate in the children of epileptic mothers exposed daily after month 4 of pregnancy, a 3·5% rate in cases where maternal exposure was sporadic during the first 4 months of pregnancy, and a 3·0% rate in children whose epileptic mothers were not exposed to the drug at all. More than half of the regular users of DPH in this survey ingested 200–300 mg/day during pregnancy. Nearly half of the remainder were on a higher dose regime, but there was no evidence that malformations were more common in infants of mothers taking the higher doses.

These workers also found, however, that the epileptic mothers tended to have certain characteristics generally associated with an increased risk of malformations. These factors included a relatively low social class and a high incidence of stillborn births and of hydramnios compared with controls. It was therefore possible that the epilepsy itself was a contributory factor in the increased incidence of malformation encountered in infants exposed regularly to DPH during early gestation. It is unlikely that epilepsy alone could explain the excess malformation rate, since the incidence of abnormalities in the offspring of epileptics not exposed to DPH did not differ statistically from that of controls. Nevertheless, since it is probably the more severe cases of epilepsy that are generally associated with regular daily treatment with the drug, some association between the malformation risk and the severity of the disease cannot be altogether discounted.

[The results of this study are in agreement with those of others previously reported (*Cited in F.C.T.* 1974, **12**, 151), indicating that the probability of malformation may be 2–3 times as great in the children of epileptics receiving diphenylhydantoin during early pregnancy as in those of non-epileptics. Further information must be obtained, however, before a sound assessment of the benefit/risk balance of this drug can finally be made.]

2764. Respiratory sensitization by a diisocyanate

Tanser, A. R., Bourke, M. P. & Blandford, A. G. (1973). Isocyanate asthma: respiratory symptoms caused by diphenyl-methane di-isocyanate. *Thorax* **28**, 596.

The hazards associated with the use of toluene diisocyanate (TDI) in the production of polyurethane materials are well documented (*Cited in F.C.T.* 1971, **9**, 279). The safety-in-use of diphenylmethane diisocyanate (MDI) is less well known, although its low volatility has led to the suggestion that it is virtually without vapour hazard (*Lancet* 1966, **i**, 32). Very few instances of respiratory sensitization have, in fact, been reported, but recent findings in workers exposed to MDI in a factory producing rigid foams make it clear that the same precautions are required for MDI as for other industrially important isocyanates.

Of 57 workers having some degree of contact with MDI, four had developed hypersensitivity to the compound. Three of these affected individuals had daily contact with MDI and the fourth, although only infrequently exposed, was known to be susceptible to bronchitis. Ten other employees experienced irritant effects, mainly of the respiratory

tract, whenever they were exposed to MDI vapour. A past history of bronchitis or allergy was found more commonly in those subjects who were obviously affected by MDI than in asymptomatic individuals, although the association was only statistically significant in the case of those with a history of allergic reactions. In this group, however, the clinical significance of the association was obscure, since only two of the seven affected workers were hypersensitive to MDI, while the others suffered only irritant effects.

The authors interpret their findings as suggesting that both immediate and delayed hypersensitivity may occur with industrial exposure to MDI. Nevertheless, even the most severely affected subjects in the present study had normal spirometric values after several months' recovery and showed no persisting symptoms.

[The absence of data relating to the levels of diphenylmethane diisocyanate to which workers were exposed in this study precludes any useful comment on the threshold limit value of 0·02 ppm set for this material by the American Conference of Governmental Industrial Hygienists. Clearly, however, the precautions required when this compound is used should be as rigorous as those that have been applied to the more notorious toluene diisocyanate for a number of years.]

2765. Hexachlorophene neurotoxicity

Towfighi, J., Gonatas, N. K. & McCree, Laura (1973). Hexachlorophene neuropathy in rats. *Lab. Invest.* **29,** 428.

In a recent article on excessive exposure to hexachlorophene (HCP), we discussed the neurotoxic effects of the germicidal agent when it is absorbed through neonatal skin (*Cited in F.C.T.* 1974, **12,** 563). In rats, focal vacuolization has been described with dietary levels of HCP in excess of 20 ppm, and levels of 50–70 ppm reduced the sciatic-nerve conduction velocity, an effect which was sometimes associated with evidence of demyelination and which cleared soon after removal of HCP from the diet (*ibid* 1974, **12,** 566). Before the significance of HCP neurotoxicity can be properly understood, it is essential to unravel the pathogenic mechanism at the ultrastructural and eventually the biochemical level. The authors cited above have begun this task by investigating by means of the electron microscope the relationship between HCP-induced peripheral-nerve oedema and subsequent demyelination in experimental animals.

HCP was administered to adult rats in the diet for periods of 2–8 wk. At the lowest dietary concentration of HCP (300 ppm) the only sign of neurological impairment was a general hind-limb weakness, but animals fed 1000 ppm HCP became paraplegic in 1–2 wk. All symptomatic animals developed a moderate to severe degree of sponginess of the white matter of the central nervous system and of peripheral nerve tissue. An examination of the ultrastructural features of the peripheral lesions showed them to stem from intramyelinic vacuolization resulting from the splitting of the myelin sheath at the intraperiod line. This vacuolization was identical to that produced in central myelin by triethyltin, cuprizone, isonicotinic acid hydrazide and HCP itself. In addition to segmental demyelination of the peripheral nerves, HCP produced abnormalities at some nodes of Ranvier, which showed segments covered asymmetrically by thin myelin layers.

The authors suggest that myelin is not metabolically stable and that HCP-induced alterations in its composition may give rise to lamellae in which the molecular organization of the sheath has been affected. The biochemical basis for the observed changes in myelin structure still remains obscure, although it is known that HCP can uncouple oxidative

phosphorylation in brain mitochondria (Cammer & Moore, *Biochem. biophys. Res. Commun.* 1972, **46,** 1887).

2766. Kerosene irritancy

Tagami, H. & Ogino, A. (1973). Kerosine dermatitis. Factors affecting skin irritability to kerosine. *Dermatologica* **146,** 123.

We have previously considered the aspiration hazard associated with ingestion of kerosene (*Cited in F.C.T.* 1974, **12,** 164), and the capacity of kerosene to irritate the skin of workers continually handling the material has been recognized for some time. Relatively few domestic cases of skin injury appear to have been reported, however.

The paper cited above is concerned with four children who showed a severe primary irritant response after exposure to kerosene and resulting contamination of their clothing. The children complained of burning and soreness, and developed lesions consisting of a well-defined erythema, scattered flaccid pustules, purulent bullae and areas of raw skin sometimes resembling a burn. The conditions were successfully treated with corticosteroids administered orally and topically.

One of the authors carried out an occlusive patch test with kerosene on himself. The response was one of intense irritation, a burning sensation shortly after application being followed by formation of a large bulla extending beyond the exposed area. This broke later, leaving a raw surface. Caucasian and negro volunteers were patch tested occlusively with various dilutions of kerosene. The degree of response was related to concentration —none of the volunteers reacted to the 40% solution, approximately 25% reacted to the 55% dilution and all reacted to the 85% dilution. Caucasian skin was more sensitive and formed pustules more readily than dark skin. Patch testing on a series of Caucasians in two age groups (21–31 and 58–82 yr) gave scattered results and no conclusion could be drawn on the possible effects of ageing on responses to kerosene, although it is known that young skin is more sensitive to irritation than that of adults (Marcussen, *Archs Derm.* 1963, **87,** 378).

Biopsy samples taken from the patch-tested areas 7 and 24 hr after kerosene application showed a histopathological picture dependent on the stage of reaction. Intra- and intercellular oedema, pyknosis, and eosinophilic cytoplasm was observed in the epidermal cells at the early (erythematous) stage, sub-epidermal slit-like spaces were seen at the tip of the dermal papillae and the dermis showed a dense perivascular lymphocytic infiltration. By 24 hr, spongiosis, exocytosis and vesicle formation were visible in the epidermis and dense lymphohistiocytic infiltration was still apparent in the upper dermis.

Because of the wide variation in individual responses to kerosene patch tests, the permeability of the stratum corneum was tested in 17 subjects by measurement of the 3,3,4,5-tetrachlorosalicylanilide solution penetration time. No clear correlation was established between skin permeability and the degree of cutaneous response to kerosene, and the authors conclude that this irritability is a function of the inherent reactivity of the tissue rather than the degree of penetration.

2767. Paraffinoma, a misleading lesion

Borrie, J. & Gwynne, J. F. (1973). Paraffinoma of lung: lipoid pneumonia. Report of two cases. *Thorax* **28,** 214.

The potential hazards of mineral oil inhalation have been the subject of a number of studies in recent years, particularly in connexion with industrial exposure to oil mists

(*Cited in F.C.T.* 1973, **11**, 925), but we have not dealt specifically with the occurrence of the localized lipoid pneumonia generally known as paraffinoma of the lung. This lesion results from the accumulation of mineral oil, or occasionally animal or vegetable oil, in the lungs. The oil is ingested by alveolar macrophages which, together with inflammatory cells and a connective-tissue response, form the lesion.

The authors of the paper cited above describe two paraffinoma cases, which were originally thought to be neoplastic on the basis of radiological examination. Thoracotomy revealed in the first case five discrete localized nodules, still thought to be neoplastic, and in the second case a single lesion, which was then thought to be tuberculous. Lobectomy, performed in both cases, was followed by complete recovery. The true nature of both lesions (paraffinoma) was only discovered on pathological examination of the excised tissue. Subsequent questioning revealed that both patients had used oily nasal drops to combat sinusitis and these were suspected of being responsible for the lesions.

[Two points emerge from this paper; firstly, more prompt questioning might have given an idea of the true nature of the lesion at an earlier stage and, secondly, indiscriminate use of oily nose drops appears to be unwise.]

2768. Methylsilicone in the lungs

Blessing, M. H. u. Lenz, W. (1973). Pathologisch-anatomische Veränderungen der Rattenlunge nach endotrachealer Injektion von Methylsilikonöl. *Int. Arch. Arbeitsmed.* **31**, 277.

The use of silicones as surgical prostheses and cosmetic aids and in food products has raised questions regarding their safety in different parts of the body, and questions have arisen, in particular, regarding events that may follow the mobilization of silicones from body deposits (*Cited in F.C.T.* 1968, **6**, 659). The effect of endotracheal application of methylsilicone, which is used industrially in sprays, has now been studied in rats.

A methylsilicone oil, derived from a commercial spray of viscosity 350 cP, was injected into the trachea of rats in single, daily or weekly doses of 0·5 ml. for up to 20 wk, the total maximum dose given being 5 ml. Animals were killed for examination after a lapse of 1–140 days from the start of treatment, and histological examination of the lungs, liver, spleen, kidneys and adrenals was carried out. In 27 of the 37 animals, no macroscopic changes were visible in these organs, apart from slight lung congestion. In ten there were pneumonic changes with occasional emphysema and atelectasis. Microscopic examination of the alveolar tissue 2 days after an injection showed macrophages with large vacuoles containing what was presumed to be the silicone. At first there were single macrophages, but afterwards they formed aggregates. There were also deposits of amorphous material in the alveolar tissue, associated with lamellar and tubular structures. There was no inflammatory reaction in the early stages, but after 6–9 wk a peribronchial leucocytic infiltration took place. Animals that survived the necrotizing pneumonia developed septal fibrosis. No changes that could be linked with storage of the silicone oil could be detected in other organs.

2769. Contraceptive organosiloxanes

Abraham, R., Fulfs, J. C., Golberg, L. & Coulston, F. (1973). Cytotoxic action of a mixed copolymer of phenylmethylcyclosiloxane on rabbit blastocyst lysosomes. *J. Reprod. Fert.* **34,** 451.

Earlier studies on an equilibrated copolymer of mixed cyclosiloxanes of the general formula $[(C_6H_5CH_3SiO)_x.([CH_3]_2SiO)_y]$, where $x + y = 3–8$ (PMCS) associated marked

gonadal effects with its dermal application, ingestion or injection (*Cited in F.C.T.* 1973, **11,** 319). In rabbits, testicular atrophy, maternal weight loss, increased resorptions and decreased viability of the young have been reported.

In the present experiments, rabbits were given 100 mg PMCS/kg by stomach tube on days 4 and 5 of pregnancy. Animals examined on days 8 and 10 showed neither blastocysts nor embryos. Blastocysts found on day 6 showed increased numbers of stained granules, some forming large aggregates, an increased number of lysosomes and a rise in acid-phosphatase activity compared with blastocysts in control animals. There were amorphous deposits, presumed to be siloxane, in trophoblastic vacuoles that were membrane-bound and lysosomal in nature. The degenerative changes induced in the trophoblast by the uptake of macromolecular material into the lysosomes evidently impeded implantation of the ovum. The uterine cells showed none of the changes usually associated with implantation, and there was no sign of the entry of siloxane into the lysosomes or cytoplasm of the uterine epithelial cells or macrophages. Since the lysosomal uptake of macromolecular material is confined to the trophoblast, this work is being extended to establish whether the viability of the blastocyst can be restored by its transfer to a favourable environment, either *in vitro* or by introduction into untreated does. Siloxanes with various chemical structures are being studied in the search for a new approach to the prevention of blastocyst implantation.

2770. PCT, a brother for PCB

Sosa-Lucero, J. C., de la Iglesia, F. A. & Thomas, G. H. (1973). Distribution of a polychlorinated terphenyl (PCT) (Aroclor® 5460) in rat tissues and effect on hepatic microsomal mixed function oxidases. *Bull. env. contam. & Toxicol.* (*U.S.*) **10.** 248.

Polychlorinated terphenyls (PCTs) have industrial applications similar to those of the more familiar polychlorinated biphenyls (PCBs), with which they are sometimes used, but they have hitherto not enjoyed the same close scrutiny. Some time ago we reviewed the toxicity of environmental PCBs (*Cited in F.C.T.* 1973, **11,** 131).

The distribution of Aroclor 5460, a PCT containing 60% chlorine, has been studied, together with its effect upon liver microsomal-enzyme activity, in rats fed 10, 100 or 1000 ppm in the diet for 7 days. All the tissues analysed (blood, brain, testes, kidney, spleen, heart, liver and body fat) contained PCT residues. The greatest concentrations (611 ppm at the highest feed level) were in the liver, while blood had the lowest concentration (5·85 ppm at the highest feed level). PCT administration did not affect body weight, but a significant increase in liver weight, both absolute and relative to body weight occurred in the rats fed 1000 ppm.

As has been shown for PCB, the constituents of the PCT with the shortest gas-chromatographic retention times, and presumably the lowest chlorine content, were metabolized to a greater degree than those with a longer retention time. Hepatic microsomal protein and phospholipids were increased by the PCT in proportion to the dose level. Glucose-6-phosphatase was diminished, but not significantly, while cytochrome *P*-450 was increased. Aniline hydroxylase and aminopyrine *N*-demethylase were both decreased by the 100 ppm level but increased by 1000 ppm. At the lowest feed level of the PCT there was no significant alteration in microsomal-enzyme activity. It is evident, at least in the male rat, that Aroclor 5460 is a potent inducer of liver microsomal enzymes when ingested in amounts constituting 100 ppm or more of the feed.

2771. Polytetrafluoroethylene in the clear

Griffith, F. D., Stephens, Susan S. & Tayfun, F. O. (1973). Exposure of Japanese quail and parakeets to the pyrolysis products of fry pans coated with Teflon® and common cooking oils. *Am. ind. Hyg. Ass. J.* **34,** 176.

The formation of toxic breakdown products of polytetrafluoroethylene (PTFE) becomes significant only at temperatures higher than those achieved in normal cooking processes (*Cited in F.C.T.* 1969, **7,** 368). Reports of deaths among cage birds after their exposure to pyrolysis products from grossly overheated frying-pans coated with PTFE prompted an investigation to determine an 'approximate lethal temperature' (ALT) for the heating of PTFE coatings, of other plastics components of cooking utensils and of various cooking materials, as judged by 4-hr exposure of Japanese quail and parakeets to the fumes emanating from the heated materials.

For Japanese quail, the ALTs for the materials tested ranged from 260°C for butter in a cast-iron pan to 370°C for the unidentified plastics handle of a glass utensil. For a frying-pan lined with PTFE the ALT was 330°C. For parakeets, the ALT for butter in a cast-iron pan or in a PTFE-lined pan was 260°C, while the ALT for a PTFE-lined pan alone was 280°C. Normal frying temperatures range from 130°C for fish to 196°C for potatoes, and the flash point for corn oil or butter is in the 260–300°C range. Thus, PTFE linings appear to be safe when used for normal cooking purposes and to offer no hazard to household pets—even to birds, which seem to be relatively sensitive to toxic pyrolysis products. Compared with the values determined in this study, the 4-hr ALT for PTFE-coated pans in rats has been reported to be 450°C. In each species studied, however, the ALT is sharply defined, passing from a no-effect range abruptly to one eliciting 100% mortality.

If severe overheating does occur, a situation that may not be totally unknown in the less well organized kitchen, it seems that some plastics handles could contribute to the liberation of toxic pyrolysis products. The practical hazards associated with such conditions, however, would be likely to extend beyond inhalation problems.

2772. Terphenyl coolants: Safe within reason

Adamson, I. Y. R. & Weeks, J. L. (1973). The LD_{50} and chronic toxicity of reactor terphenyls. *Archs envir. Hlth* **27,** 69.

It has been reported that fresh hydrogenated terphenyl mixtures intended for coolant purposes in nuclear reactors are free from significant carcinogenic potential when applied to the skin, and after heating and irradiation they remain only mildly carcinogenic compared with tar (*Cited in F.C.T.* 1973, **11,** 1153). Moreover, it has been found that cell renewal in the lung and adaptation in the liver of mice compensates for any effect terphenyls may have when inhaled at levels well above the practical levels of industrial exposure (*ibid* 1974, **12,** 284). We now have data on the effects of ingesting a coolant of this type.

The acute LD_{50} of non-irradiated and irradiated samples of a coolant (HB-40) consisting of *o*-, *m*- and *p*-terphenyls with a small proportion of higher polymers was determined in mice and rats by intragastric injection of single doses, initially in the range of 1000–50,000 mg/kg and subsequently in a narrower range around the possible LD_{50}. Chronic toxicity was determined in mice by administering 20–2000 mg terphenyls/kg by gastric intubation daily for up to 16 wk. Lethal doses of non-irradiated HB-40 killed in 3–4 days, while irradiated HB-40 produced maximum mortality in 1–2 days. There was no obvious

cause of death in either case. The LD_{50} of non-irradiated HB-40 was about 17,500 mg/kg in rats and 12,500 mg/kg in mice, and the value of irradiated HB-40 was about 6000 mg/kg in both species. The first pathological effect of the chronic ingestion of HB-40 was kidney damage, both samples producing qualitatively similar lesions which progressed with increasing exposure. The shape and arrangement of mitochondria in the tubular epithelial cells were altered, and necrosis of these cells occurred later. High doses (1200 and 600 mg/kg for non-irradiated and irradiated HB-40 samples, respectively) given daily for 16 wk produced irreversible interstitial nephritis with scarring. Liver toxicity was evident only at the ultrastructural level, with proliferation of the smooth endoplasmic reticulum.

From these findings it is evident that terphenyl coolants are toxic by ingestion, but only in very high doses unlikely to be encountered in reactor operations.

2773. Trichloroethylene: A serious complication of exposure

Sagawa, K., Nishitani, H., Kawai, H., Kuge, Y. & Ikeda, M. (1973). Transverse lesion of spinal cord after accidental exposure to trichloroethylene. *Int. Arch. Arbeitsmed.* **31,** 257.

Neurotoxic effects of trichloroethylene (TCE) have received much attention from industrial physicians. Toxic effects on the peripheral nervous system have been attributed to the TCE metabolite, trichloroethanol (*Cited in F.C.T.* 1974, **12,** 163), and direct neuronal lesions have been reported in rabbits (*ibid* 1971, **9,** 755). The paper cited above gives a grimmer picture of the possibilities of industrial over-exposure to TCE.

A young woman was accidentally exposed at work to TCE vapour from a vapour degreasing bath when the cooling system failed. Part of the solvent was trapped and liquefied in a suction duct, falling as a fine rain. The patient initially experienced abdominal discomfort, nausea, vomiting and a feeling of inebriation, followed by sudden cramps in the extremities. She lost consciousness for 2 hr, and subsequently demonstrated anaesthesia to touch and pain below the fifth thoracic level. About 1 wk later she complained of increasing headache, nausea and vomiting. Her gait was unstable and she became unable to stand or walk unaided. Other abnormalities included a reduction in visual acuity, urinary retention, constipation and sphincter disturbances. During the next 2 months there was a slow partial recovery, but treatment with vitamin B complex, corticosteroids and vasodilators failed to eliminate the residual disability. Physiotherapy for 9 months brought only slight restoration of mobility. A second woman involved in the same accident suffered a less severe degree of exposure to TCE and was unconscious for 1 hr, subsequently complaining of headache, retrobulbar pain, nausea and inability to concentrate. Most of her neurological disturbances resolved within 4 months, but occipital headache and proneness to motion sickness were more persistent.

From a consideration of the urinary concentration of trichloro compounds found in the first patient, she was estimated to have been exposed to a narcotic TCE concentration of several thousand ppm at the time of the accident. Since the severity of the neurological effects was uncharacteristic of TCE itself, it is possible that unidentified decomposition products in the repeatedly recycled TCE, or added stabilizers or inhibitors added to prevent solvent decomposition, may have been responsible. No direct evidence of the nature of the spinal cord lesions was obtained, but the clinical findings strongly suggested an extensive transverse lesion of the spinal cord at the lower cervical to upper thoracic level.

NATURAL PRODUCTS

2774. Olive oil and the reticulo-endothelial system

Berken, A. & Sherman, A. A. (1972). Reticuloendothelial system depression in man after olive oil ingestion. *Proc. Soc. exp. Biol. Med.* **141,** 656.

The ingestion of various lipids, including olive oil, has been shown to depress phagocytosis of colloidal carbon by the reticulo-endothelial system (RES) of mice (Berken & Benacerraf, *Proc. Soc. exp. Biol. Med.* 1968, **128,** 793). In man, colloidal carbon uptake is not an appropriate means of measuring the phagocytic function of the RES. Instead, this function can be measured by the disappearance from the blood of a radio-iodinated microaggregate of human serum albumin, which is taken up readily by the human RES. This method has been used to establish whether olive oil has the same depressant effect on the RES system in man as it has in the mouse.

Over a 24-hr period, ten male and two female patients drank 24 oz of a flavoured aqueous emulsion containing 8 oz commercial olive oil. The oil was sufficiently palatable to be consumed, in approximately 4 oz portions, "without objection if not with great relish", and no gastro-intestinal distress ensued. Three hours after consuming the last of the oil, each patient was given [^{125}I]-labelled microaggregated serum albumin in the form of a mixture containing 25 mg albumin/ml and having an activity of 4–5 μCi/ml. The dose, given by iv injection, was equivalent to 2·5 mg albumin/kg, and blood samples were taken 5, 10, 15, 20 and 25 min later. The radioactivity of these samples was counted and the plasma clearance of the radioactive albumin was compared with that determined for the same patients under a comparable regime of iv dosing and blood sampling, 1 wk before ingestion of the oil.

Depression of the phagocytic index was noted in all patients after oil ingestion, but the degree of change was variable. Those with the highest clearance rates before oil administration showed the greatest depression. A considerable increase in serum triglyceride levels was another result of the olive oil ingestion.

[This piece of work is particularly interesting in view of the current concern about the possible consequences of depression of the reticulo-endothelial system as a result of ingestion of macromolecules. Daily intakes of oil of the order of that consumed in this study are common in some Mediterranean countries, yet in these areas there seems to be no more deficiency in the immunological response than in countries in which the quantities of olive oil consumed are much smaller.]

2775. Peanuts with a difference

Alfin-Slater, R. B., Wells, P., Aftergood, L. & Melnick, D. (1973). Safety of nuts heat-processed in molten hexitols. *J. Am. Oil Chem. Soc.* **50,** 348.

A blend of the hexitols, mannitol (m.p. 166–168°C) and sorbitol (m.p. 89–101°C) in the proportion of 80:20 parts (w/w) has been used in place of frying-fat to produce the so-called dry-roasted peanuts. This dry-roasting procedure is claimed to have some advantages over the conventional air-roasting process, particularly in that the hexitols, while behaving like solid fats, are not susceptible to oxidative deterioration.

Peanut butter derived from hexitol-roasted nuts was compared with butter from air-roasted nuts when fed at a dietary level of 35% to rats over four generations. Rats given the conventional product showed a slightly greater rate of weight increase than those given

hexitol-roasted nuts, although food utilization in the latter group was satisfactory and overall weight gain was not depressed. There was no increase in morbidity attributable to the hexitols, and no diarrhoea occurred. The hexitol-roasted product apparently improved breeding performance slightly compared with the control product. The pathological findings and organ weights did not differ significantly between the two groups. The group on hexitol-roasted nuts had somewhat lower total liver lipid and cholesterol levels than the rats on the conventional nut product.

The findings in the group given the conventional peanut butter in the diet were comparable to those recorded for diets of similar composition but containing no peanut-derived components. This test therefore yielded no evidence for the production of any toxic factor in peanuts processed either in the conventional way or by dry-roasting. As far as sorbitol is concerned, the lack of formation of any toxic factor in the peanut-roasting process, which involved temperatures of 150–170°C, is not surprising, since no harmful degradation products have been detected either in the production of the approved food additive, sorbitan monostearate, by reaction of sorbitol with fatty acid for several hours at 220°C or in the heating of sorbitol to 200°C in the preparation of certain candies.

2776. Sterigmatocystin in country-cured ham?

Halls, N. A. & Ayres, J. C. (1973). Potential production of sterigmatocystin on country-cured ham. *Appl. Microbiol.* **26,** 636.

Reference has previously been made to the production of ochratoxins A and B by *Aspergillus ochraceus* on country-cured hams (*Cited in F.C.T.* 1974, **12,** 287). In a survey of the mycoflora of such hams, Sutic *et al.* (*Appl. Microbiol.* 1972, **23,** 656) found that moulds in the *A. versicolor* group were the second most common aspergilli. Certain strains of *A. versicolor* produce sterigmatocystin, a recognized hepatocarcinogenic mycotoxin with a carcinogenic potency not far behind that of aflatoxin B_1 (Purchase & van der Watt. *Fd Cosmet. Toxicol.* 1968, **6,** 555).

Of 16 strains of *A. versicolor* isolated from country-cured ham and cultured on three types of laboratory media for 7, 10 or 14 days, ten were found to be capable of producing sterigmatocystin. Three of these isolates were cultured on 25-g slices of country-cured ham at 20 or 28°C for periods of up to 14 days. After 7 days, no sterigmatocystin was detected (limit of detection, 0·5 μg/slice) on any slice of ham stored at 28°C and it was found only on one slice kept at 20°C, but after 14 days, levels of 4–20 μg/slice were detected on all slices except one incubated at 20°C. No mould growth was apparent on uninoculated control slices of ham.

The authors concluded that although there was no direct evidence that country-cured hams contained sterigmatocystin, they could provide an environment in which a toxigenic mould such as *A. versicolor* could be a hazard to the health of the consumer.

2777. Factors in mycotoxin production

Bacon, C. W., Sweeney, J. G., Robbins, J. D. & Burdick, D. (1973). Production of penicillic acid and ochratoxin A on poultry feed by *Aspergillus ochraceus*: Temperature and moisture requirements. *Appl. Microbiol.* **26,** 155.

Two strains of *Aspergillus ochraceus*, isolated during investigations of toxigenic mycoflora in poultry feed, were found to produce penicillic acid and ochratoxin A. The interaction of moisture and temperature factors on the production of these two metabolites was

complex. Low temperatures (15–22°C) combined with a fairly low moisture content (23%) favoured production of penicillic acid, whereas at 30°C and a high humidity, ochratoxin A production was favoured. At temperatures between 22 and 30°C, both mycotoxins were produced to a marked degree, but while there was a clear drop in penicillic acid production with moisture levels from 42% and upwards, the decline in ochratoxin A yields did not begin until a moisture level as high as 52% was reached.

COSMETICS, TOILETRIES AND HOUSEHOLD PRODUCTS

2778. Aerobic microbial breakdown of secondary alkyl sulphates

Lijmbach, G. W. M. & Brinkhuis, Elisabeth (1973). Microbial degradation of secondary *n*-alkyl sulfates and secondary alkanols. *Antonie van Leeuwenhoek* **39**, 415.

Primary *n*-alkyl sulphate detergents have become widely used because they are largely biodegradable, their breakdown proceeding via the alcohol and aldehyde to the acid, which is converted to carbon dioxide and water by β-oxidation (Williams & Payne, *Appl. Microbiol.* 1964. **12,** 360). Considerably less has been known about the biodegradability of secondary *n*-alkyl sulphate detergents, particularly under anaerobic conditions. Many bacteria possess sulphate-hydrolysing esterases, so the first reaction of the biodegradation sequence, leading to the formation of a secondary alcohol, should proceed readily. The paper cited above describes a study of the route of bacterial breakdown of the resulting secondary alcohols, using 2-butanol as a model compound.

The bacterium used, a species of Pseudomonas, was isolated from harbour water and was able to grow aerobically on a mineral medium containing secondary alcohols as the sole carbon source. Attempts to isolate bacteria that would utilize the secondary alcohol anaerobically were unsuccessful. Growth of the pseudomonad on 2-butanol sequentially induced a number of enzymes which catalysed the conversion of the intermediate oxidation products of 2-butanol, namely 2-butanone, 3-hydroxy-2-butanone (acetoin) and 2,3-butanedione (diacetyl). Using cell-free extracts of these bacterial cells, it was possible to convert 2-butanol or 2-butanone to acetoin, diacetyl and acetic acid in the presence of $NADH_2$. In addition, 2-butanone[2-^{14}C] was converted to labelled acetoin by the cell-free extract. These findings suggest the following pathway for the bacterial degradation of secondary alcohols: Alcohol → ketone → hydroxyketone → dione → aldehyde + acid, followed by β-oxidation of the resulting fatty acids to carbon dioxide and water. This pathway has been shown to hold for other long-chain secondary alcohols, notably 2-pentanol, 2-octanol, 3-hexanol, 3-nonanol and 3-, 4- and 5-decanols.

2779. Sodium lauryl sulphate under attack

Bergstresser, P. R. & Eaglstein, W. H. (1973). Irritation by hydrophilic ointment under occlusion. *Archs Derm.* **108,** 218.

The skin irritancy of sodium lauryl sulphate is well established, but little attention has apparently been paid to the possibility that its use as an emulsifier in a preparation for topical application might cause a dermatitic reaction. In the work cited above, three formulations were tested, two containing 1% sodium lauryl sulphate and one containing 2% polyoxyethylene (40) stearate instead. One of the formulations containing sodium lauryl sulphate was hydrophilic ointment (US Pharmacopeia, 14th revision, Washington, D.C., 1950, p. 391) and the other two creams were similar in composition. The substances were

applied under occlusion to hospital patients and staff for two periods of 8 hr each day. Dermatitis was reported after 3–5 days in all subjects in response to the formulations containing sodium lauryl sulphate, but no reaction was observed after 7 days of treatment with the third formulation. The possibility that the reactions might have been due to contact sensitivity was eliminated by standard patch testing. The authors concluded that less irritant emulsifiers than sodium lauryl sulphate or concentrations of this emulsifier below 1%, should be used in the formulation of topical products.

[The test conditions described here, however, seem to be much more severe than the normal conditions of use of most topical products, and thus the authors' conclusion may not be wholly justified. The fact that most of the subjects had some form of skin disorder at other sites adds a further cautionary note.]

2780. Surfactants and skin permeability

Dugard, P. H. & Scheuplein, R. J. (1973). Effects of ionic surfactants on the permeability of human epidermis: An electromagnetic study. *J. invest. Derm.* **60,** 263.

It has already been established that various ionic surfactants applied in aqueous solution increase the permeability of human skin (*Cited in F.C.T.* 1966, **4,** 88; *ibid* 1969, **7,** 191). An *in vitro* comparison has now been made of the effects of different surfactants on the permeability of human cadaver epidermis previously stripped by heat treatment. The three C_8–C_{16} straight-chain series of ionic surfactants used were sodium *n*-alkyl sulphates (R-OSO_3Na), sodium salts of primary aliphatic acids (R-COONa) and *n*-alkylamine hydrochlorides (R-NH_3Cl). So that electrical conductance could be used to indicate permeability, the relationship between the two properties was established with C_{12} surfactants using tritiated water to measure permeability. Since the graphs of permeability against conductance measured during progressive surfactant action were all approximately linear, it was concluded that conductance provided a reasonable measure of permeability.

Using this technique, therefore, to study the effect of molecular size on permeability, the authors found that with all three series, the maximum increase in conductance occurred with C_{12} and C_{14} chain lengths, while C_{10} and C_{16} compounds caused little increase and C_8 compounds had no effect. Increase in surfactant concentration from the 5·0 mM concentration used for most of the study increased the rate of change in conductance, but alterations in pH of the C_{12} members of each series did not affect this rate. The rates of increase were similar for the two anionic series, but more rapid increases shortly after contact were found with the alkylamine hydrochlorides. The reversibility of surfactant effects on permeability was more complete with shorter periods of exposure.

The authors discuss these results in relation to the question of whether the primary action of surfactants is on the protein or the lipid component of the keratin layer. They conclude that since the effect on permeability is not clearly related to the detergent action, interaction with the protein component is probably more important.

2781. Searching for sensitizers in vaginal deodorants

Fisher, A. A. (1973). Allergic reaction to feminine hygiene sprays. *Archs Derm.* **108,** 801.

Vaginal deodorants may sometimes provoke adverse and occasionally fairly severe skin reactions. The responsible ingredients have not been clearly identified, although hexachlorophene, perfumes and propellants have been implicated as probable causes (*Cited in F.C.T.* 1973, **11,** 342). Hexachlorophene has now been withdrawn from use in such pro-

ducts, but among the bacteriostats still sometimes used are chlorhexidine, which is a rare sensitizer (*ibid* 1973, **11,** 166), and benzethonium chloride, which is also occasionally guilty in this respect (*ibid* 1973, **11,** 1157).

The ingredients of 12 different sprays which had produced a vulvar and inguinal or thigh dermatitis have now been patch-tested on 30 affected women and also on two men, who had suffered penile and scrotal dermatitis as a result of coitus immediately after their partner's use of one of these sprays. Only five positive reactions were obtained, two (in one male and one female) to 0·1% benzethonium chloride, one to 1% chlorhexidine (associated with the use of a spray purchased in Sweden), one to isopropyl myristate and one, in a woman with a known perfume sensitivity, to the perfume component. In two of these cases the allergic reaction was superimposed on an existing dermatological condition.

Isopropyl myristate is rarely a sensitizer (Fisher *et al. Archs Derm.* 1971, **104,** 286), although six cases of allergy from its use in an antibiotic-steroid spray have previously been reported (Calnan, *Proc. R. Soc. Med.* 1962, **55,** 39). Patch tests with the bacteriostats triclosan (Irgasan DP 300) and chloroxylenol, which are not now used in sprays manufactured in the United States, were negative in all cases. In the 27 subjects in whom an allergic cause could not be identified, the reactions were assumed to have been due to the chilling and irritating effects of the fluorocarbon propellants. Such effects result when, despite the presence of labels on all products specifically warning against this, the spray is held too close to the body.

[Although it is reassuring to find that adverse reactions to such products are now largely the result of misuse, it is evident that such misuse can occur with ease, and more emphatic warning labels would seem to be necessary if products for this purpose are to continue to be marketed.]

PATHOLOGY

2782. A new line on hepatic disorders

Smith, M. G. M., Williams, R., Walker, G., Rizzetto, M. & Doniach, Deborah (1974). Hepatic disorders associated with liver/kidney microsomal antibodies. *Br. med. J.* **2,** 80.

The hepatic microsome has been closely associated with a group of enzymes, usually called the 'mixed-function oxidases', which are involved in the processing of many foreign compounds and which often show an increase in activity when pathological changes develop in other organs. Interest in the microsome has now extended from the field of enzymology to that of immunology, with the development of a technique by which it has proved possible to demonstrate an antibody to hepatic microsomes. This antibody also reacts with renal microsomes and is known as the liver/kidney microsomal (LKM) antibody.

Testing of all samples of sera sent to a particular London laboratory over a given period revealed that 33 patients (16 male and 17 female) had LKM antibodies. This group, which represented 0·12% of the total number tested, included 15 patients with active chronic hepatitis and seven cases of acute hepatitis, three of the latter cases being attributed to a virus A infection and four to hypersensitivity to drugs. The remaining cases included two with sub-clinical hepatitis and two with hepatocellular carcinoma. No other antibodies (antinuclear, smooth muscle or antimitochondrial antibodies) were detected in the

sera of these patients. This suggests that active chronic hepatitis may represent a distinct sub-group of liver disease.

[This is an important step forward in the differential diagnosis of hepatic disorders. It opens up an interesting field of speculation for the toxicologist interested in the significance of the hypertrophy of smooth endoplasmic reticulum often produced by compounds that induce liver enlargement. One wonders whether such hypertrophy could possibly predispose to active chronic hepatitis.]

Fd Cosmet. Toxicol. Vol. 12, p. 803. Pergamon Press 1974. Printed in Great Britain

MEETING ANNOUNCEMENT

FRENCH SOCIETY FOR LABORATORY ANIMAL SCIENCE

The VIth Annual Meeting of the French Society for Laboratory Animal Science will be sponsored by the Paris-Val de Marne University and will be held at the University of Paris-Val de Marne Medical School, 94000 Creteil, on 31 May 1975. The main subject of the meeting will be "Animal Models of Human Pathology".

Further information about the meeting may be obtained from: Prof. J.-C. Friedmann or Mlle M. C. Trocars, Centre d'Etudes Biologiques I.F.M., BP 309, 27005 Evreux, France (Telephone: 16/32 39.26.26).

Fd Cosmet. Toxicol. Vol. 12, p. 805. Pergamon Press 1974. 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*:

Short-term toxicity of Yellow 2G in pigs. By I. F. Gaunt, K. R. Butterworth, P. Grasso and Jean Hooson.

Monosodium glutamate studies in four species of neonatal and infant animals. By B. L. Oser, K. Morgareidge and S. Carson.

Short-term toxicity of dimethyl sulphide in the rat. By K. R. Butterworth, F. M. B. Carpanini, I. F. Gaunt, Joan Hardy, Ida S. Kiss and S. D. Gangolli.

Studies on the metabolic fate of ^{32}P-labelled Emulsifier YN in the mouse, guinea-pig and ferret. By J. C. Phillips, I. F. Gaunt and S. D. Gangolli.

Long-term toxicity of sorbic acid in the rat. By I. F. Gaunt, K. R. Butterworth, Joan Hardy and S. D. Gangolli.

Effect of paraquat on the biosynthesis of deoxyribonucleic acid, ribonucleic acid and protein in the rat. By G. K. Van Osten and J. E. Gibson.

Mutagenicity studies with captan, captofol, folpet and thalidomide. By G. L. Kennedy, Jr., D. W. Arnold and M. L. Keplinger.

Mutagenicity studies with δ-aminolaevulinic acid. By D. W. Arnold, G. L. Kennedy, Jr., M. L. Keplinger and J. C. Calandra.

Effects of hexachlorophene on developing rats: Toxicity, tissue concentrations and biochemistry. By A. G. Ulsamer, D. Yoder, R. D. Kimbrough and F. N. Marzulli.

Percutaneous penetration of hexachlorophene as related to receptor solutions. By D. W. C. Brown and A. G. Ulsamer.

Ipomeamarone, a toxic furanoterpenoid in sweet potatoes (*Ipomea batatas*) in the United Kingdom. By D. T. Coxon, R. F. Curtis and Barbara Howard. (Short Paper).

Monographs on fragrance raw materials. By D. L. J. Opdyke.

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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.

Some other Pergamon Journals which may interest readers of *Food and Cosmetics Toxicology:*

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Journal of Aerosol Science

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e.g. Hickman, J. R., McLean, D. L. A. & Ley, F. J. (1964). Rat feeding studies on wheat treated with gamma-radiation. I. Reproduction. *Fd Cosmet. Toxicol.* **2**, 15.

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