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Index of Authors

The Index of Authors contains the names of all authors of original papers, review articles and Letters to the Editor. For Reviews of Recent Publications, Book Reviews and Abstracts, only the names of all the authors mentioned in the reference are given. For Articles of General Interest the names of all the authors mentioned in the text are provided, including those constituting the words *et al.*

The various features of the Journal are distinguished by the use of the following superscripts inserted after the appropriate page number:

P = Papers (original); M = Monographs; R = Review papers; L = Letters to the Editor. S = Summaries of toxicological data.

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*The significance of the superscripts P, M, R, L and S is given in paragraph 2 of the Index of Authors (p. 3).

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INFORMATION SECTION

ARTICLES OF GENERAL INTEREST*

TOXICOLOGY: ABSTRACTS AND COMMENTS*

* Except where indicated otherwise, 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.

† By G. J. van Esch and M. J. van Logten, National Institute of Public Health, Bilthoven, The Netherlands.

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Research Section

SHORT-TERM TOXICITY OF YELLOW 2G IN PIGS

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(*Received 8 March 1974*)

Abstract—Yellow 2G was given to groups of three pigs of each sex at doses of 0 (control), 5, 50 or 500 mg/kg/day for 15 wk. The faeces of the pigs on the two higher levels of treatment developed a reddish colour on exposure to the air, probably as a result of oxidation of a metabolite of the colouring. There was an initial diarrhoea lasting 1 or 2 days in half the pigs given the highest dose level. No adverse effects were seen in the rate of body-weight gain, haematology, examination of urine, organ weights or histopathological examination. The no-untoward-effect level established was 500 mg/kg/day.

INTRODUCTION

Yellow 2G (C.I. (1956) no. 18965) is the disodium salt of 1-(2,5-dichloro-4-sulphophenyl)-5-hydroxy-3-methyl-4-(*p*-sulphophenylazo)pyrazole.

It is permitted for use in food in the UK under the Colouring Matter in Food Regulations 1966 (Statutory Instrument 1966, no. 1203) but is not permitted in the EEC. It may continue to be used in the UK until December 1977, but use beyond that date will be subject to discussion within the EEC. The Food Standards Committee (1964) in its Report on Colouring Matters and the Joint FAO/WHO Expert Committee on Food Additives (1965) pointed out that there was a lack of toxicological information on this colouring.

In rats, 95–100% of an iv dose is excreted unchanged in the bile within 6 hr, showing that Yellow 2G is not metabolized in the liver (Ryan & Wright, 1961). This lack of degradation by the liver was confirmed in *in vitro* studies, in which only 17% of the colour was reduced by rat-liver homogenate (Ryan, Roxon & Sivayavirojana, 1968). However, it seems likely that its metabolism is similar to that of the chemically related tartrazine, which is the trisodium salt of 1-(4-sulphophenylazo)-5-hydroxy-3-carboxy-4-(*p*-sulphophenylazo)pyrazole.

In the case of tartrazine, Jones, Ryan & Wright (1964) showed that azo reduction, with liberation of sulphanilic acid, occurred after oral administration in the rat, rabbit and man. In addition, they produced evidence to show that this reduction took place in the intestine but not in the liver. Westöö (1965) confirmed this metabolic fate in rats and showed that the reduction occurred in the caecum or colon, as the metabolic products were not present in the small intestine. He also found that the 4-amino-5-oxo-1-(*p*-sulphophenyl)-2-pyrazoline-3-carboxylic acid metabolite excreted in the faeces is unstable in air and changes from colourless to purple on oxidation. Gaunt, Carpanini, Kiss & Grasso (1971) showed a similar colour change in the faeces of rats given oral doses of Yellow 2G, again suggesting that its metabolism was likely to be similar to that of tartrazine.

There were no adverse effects, apart from a self-limiting diarrhoea, in rats fed for 90 days on diets containing up to 1000 ppm Yellow 2G. At a higher level (10,000 ppm) the caecum was enlarged (Gaunt *et al.* 1971). In order to extend the information available concerning the toxicity of Yellow 2G, the present short-term study was carried out in pigs as part of the BIBRA safety evaluation programme.

EXPERIMENTAL

Materials. The Yellow 2G used was supplied through the Chemical Industries Association and complied with the following British Standards Institution (1963) specification:

> Dye content, min. 85%; matter volatile at 135 C*, max 10%; matter insoluble in water*, max 0·1%; matter soluble in diisopropyl ether*, max 0·2%; subsidiary dyes, max 2%; chloride and sulphate (as sodium salts)*, max 5%; copper*, max 10 ppm; arsenic*, max 1 ppm; lead*, max 10 ppm; heavy metals (as sulphides)*, producing a colour no more intense than that of the reference standard.

Animals and diet. Three litters, each of four male and four female 10-wk-old pigs of the Large White strain, obtained from a minimal-disease herd, were used. The basal diet was Hi-lean Rearers Pencils (British Oil and Cake Mills Ltd., London) fed at 0·5–1·5 lb day depending on the age of the pigs. Water was given *ad lib.*

Experimental design and conduct. The pigs were allocated to four groups of three males and three females, so that each group contained one pig of each sex from each of the three litters. These groups were fed Yellow 2G at dose levels of 0 (control), 5, 50 or 500 mg kg day for 15 wk. The required quantity of colouring was mixed with part of the daily feed, syrup (Tate and Lyle Ltd.) was added and this mixture was given before the main feed. In this way, the dose was rapidly consumed.

The animals were weighed initially and thence weekly throughout the experimental period. Blood was collected from an ear vein after treatment for 6 wk and from the anterior vena cava prior to autopsy. The blood was examined for haemoglobin concentration, packed cell volume and counts of erythrocytes, reticulocytes, total leucocytes and the different types of leucocyte. Urine samples were collected from each pig over a 2-hr period during wk 6 and 14 and measurements were made of the specific gravity. The sediment was examined microscopically and the content of glucose, blood, bile and ketone bodies was determined.

The pigs were killed by exsanguination under barbiturate anaesthesia and an autopsy was conducted, during which the brain, pituitary, thyroid, heart, lungs, liver, spleen, kidneys, adrenal glands, gonads, stomach, small intestine and caecum were weighed. Samples of these organs and of the tongue, oesophagus, trachea, thymus, lymph nodes, colon, rectum, pancreas, aorta, diaphragm, skeletal muscle, sternum, urinary bladder, ureters, urethra, seminal vesicle, vas deferens, prostate, uterus, Fallopian tubule and vagina were preserved in buffered formalin. Paraffin-wax sections of these tissues were stained with haematoxylin and eosin for microscopic examination.

RESULTS

There were no abnormalities in the condition or appearance of the pigs, except that diarrhoea was seen for the first 2 days of treatment in two male pigs and one female at

*By methods of analysis described in BS 3210: 1960 (Methods for the Analysis of Water-soluble Coal-tar Dyes Permitted for Use in Foods).

the highest treatment level (500 mg/kg/day). Throughout the study, the faeces of the treated pigs were normal in colour when freshly passed. However, in the group given the highest level of Yellow 2G, the faeces were markedly red on standing. At the lowest level (5 mg/kg/day) no colour change was seen and with the intermediate dose (50 mg/kg/day) the change was sufficient to darken the faeces. Tests for blood in the faeces were negative.

There were no dose-related adverse effects on body-weight gain, and even in cases where the mean gain of the treated group was less than that of the controls (notably in females on 5 mg/kg/day during the last month of the test), a comparison of the litter mates showed that the effect was not evident in all three pigs.

No adverse effects were detected in the haematological examinations (Table 1). The specific gravity of the urine and the composition of the urinary sediment were similar in all the pigs. No abnormal constituents were found in the urine except for a red colour at the highest dietary level. Tests for blood were negative. The method of collection did not fully preclude leaching of the faeces and no similar colour was found in urine taken directly from the bladder at autopsy.

Table 1. *Haematological findings in pigs given 0–500 mg Yellow 2G/kg/day for 15 wk*

Dose level (mg/kg/day)	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	12·8	40	6·85	0·5	15·36	26	1	71	2
5	12·7	40	7·74	0·6	15·56	24	0	74	2
50	12·9	42	7·69	0·9	13·42	28	1	70	1
500	13·5	44	7·56	0·6	14·49	31	2	65	2
Females									
0	13·0	41	7·46	0·7	15·33	28	2	68	2
5	13·5	40	7·87	0·8	14·76	30	1	67	2
50	13·1	41	7·41	0·7	13·15	32	1	65	2
500	13·2	42	7·21	0·5	13·41	31	2	64	3

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

Figures are means for groups of three pigs. No differences between treated and control animals were found at wk 6. No intra-erythrocytic inclusions were seen at wk 6 or 15 and basophils did not account for more than 0·5% of the leucocytes in any group.

There were scattered statistically significant differences between the organ weights of control and treated pigs (Table 2). In general these changes were not dose-related and were seen in one sex only. No abnormal findings were present in any of the tissues examined at autopsy or in the histopathological examination.

DISCUSSION

The observation that the faeces turned red on exposure although they were of normal colour when freshly passed, is in keeping with the findings in rats (Gaunt *et al.* 1971). It is similar to the effect seen after the feeding of the chemically related tartrazine (Westöö, 1965) and suggests that the metabolic fate of the two compounds is similar. It is likely that

Table 2. *Organ weights and relative organ weights of pigs given 0–500 mg Yellow 2G/kg/day for 15 wk*

Sex and dose level (mg/kg/day)	Weights of Brain	Heart	Lungs	Liver	Spleen	Kidneys	Stomach	Small intestine	Caecum	Adrenals†	Gonads‡	Pituitary§	Thyroid†	Terminal body weight (kg)
							Organ weight (g)							
Male														
0	97	145	219	597	36	96	293	850	78	2·48	54	120	2·89	32·5
5	98	122*	190	680	30	86	328	977	63	2·02	78	133	2·34	31·7
50	97	141	247	747	37	82**	330	957	69	2·18	75	133	2·47	35·2
500	94	132	205	602	31	86	312	867	65	1·97	75	130	2·64	33·7
Female														
0	100	156	234	669	42	94	359	1141	76	2·48	4·10	173	3·15	37·8
5	101	145	220	713	36	93	333	1022	63	2·18	4·08	230	2·66	36·2
50	99	143	225	839	37	96	351	1128	78	2·47	3·89	200	2·49	38·0
500	100	134*	232	689	41	98	373	1043	73	2·34	3·20	207	2·95	38·2
							Relative organ weight (g/kg body weight)							
Male														
0	3·08	4·49	6·76	18·5	1·10	2·97	9·08	26·3	2·44	77	1·66	3·7	81	
5	3·11	3·84*	6·00	21·6	0·94	2·70	10·34	30·9*	1·97	64	2·46	4·2	74	
50	2·75*	4·07	7·00	21·2	1·07	2·34**	9·38	27·2	1·91	62	2·16	3·8	70	
500	2·83	4·13	6·29	17·9	0·93	2·57	9·38	26·4	1·98	59	2·19	3·9	78	
Female														
0	2·68	4·13	6·23	17·7	1·09	2·48	9·54	30·3	2·02	66	108	4·6	84	
5	2·79	3·99	6·05	19·8	1·01	2·58	9·17	28·3	1·74	60	114	6·4*	73	
50	2·62	3·76	5·91	22·0	0·97	2·54	9·22	29·8	2·05	65	103	5·2	66	
500	2·61	3·51*	6·08	18·1	1·08	2·57	9·78	27·3	1·91	61	83	5·4	78	

† Relative weights of these organs are expressed in mg/kg body weight.
‡ Relative weights of ovaries are expressed in mg/kg body weight.
§ Weights and relative weights of this organ are expressed in mg and mg/kg body weight respectively.

The figures are means for groups of three pigs and those marked with asterisks differ significantly (test of Lord, 1947) from those of the appropriate control group: $*P < 0{\cdot}05$; $**P < 0{\cdot}01$.

the metabolism involves a reduction of the azo link, probably in the lumen of the gastro-intestinal tract, as has been shown to occur with tartrazine (Jones *et al.* 1964; Westöö, 1965). The sulphanilic acid is probably largely absorbed and excreted in the urine, while the substituted phenylpyrazole remains in the faeces.

In the present study, no untoward effects were found in the pigs given doses of up to 500 mg Yellow 2G/kg/day for 15 wk. The changes in organ weight were scattered, were not dose-related and were not seen in both sexes. Since no changes were seen in the histopathological examination, the variations in organ weight could not be attributed to the feeding of the colouring.

Caecal enlargement and transitory diarrhoea were found in rats fed diets providing intakes of 80 or 800 mg/kg/day (Gaunt *et al.* 1971). In the present study diarrhoea was seen only during wk 1 in the group given the highest level of colouring and only in half of the animals. No effect on caecal size or weight was evident and the transitory diarrhoea was not sufficient evidence to suggest that the Yellow 2G was exerting a toxic effect.

Thus, in this study, the no-untoward-effect level of Yellow 2G was 500 mg/kg/day, a level considerably higher than the 80 mg/kg/day found in rats (Gaunt *et al.* 1971).

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MONOSODIUM GLUTAMATE STUDIES IN FOUR SPECIES OF NEONATAL AND INFANT ANIMALS

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(*Received 28 April 1974*)

Abstract—As an extension of our previous work, the effect of both intragastric and sc administration of monosodium glutamate (MSG) was studied in four species of neonatal animals (mice, rats, beagle dogs and cynomolgus monkeys). Control groups were dosed with sodium chloride, sodium gluconate, potassium glutamate and distilled water. A uniform dose level of 1 g/kg body weight (10 ml of a 10% aqueous solution/kg) was used. Animals were killed at 3, 6 or 24 hr (and in the case of dogs also at 52 wk) after dosage. Several monkeys were also dosed orally with MSG or sodium chloride at 4 g/kg.

No adverse effect was observed on growth, appearance or behaviour in any of the species. Examination of multiple sections of the brain, eyes and pituitaries by light microscopy revealed no pathological alteration. Failure to observe in the arcuate nuclei of the hypothalamus the lesion described by Olney (*Science, N.Y.* 1969, **164**, 719) is discussed, in relation particularly to the 1 g/kg dosage level, which is many times the estimated maximum human dietary level.

INTRODUCTION

Olney (1969) initially reported a neuropathological effect of monosodium glutamate (MSG) upon the brain of the mouse. Although lesions were found in adult mice given sc doses of 5–7 g/kg, infant mice were more sensitive. Mice of 2–9 days of age were given a single sc injection (dosage ranging from 0·5 to 4 g/kg body weight) and were killed 1–48 hr after dosing. Light-microscopic examination revealed acute neuronal necrosis (intracellular oedema and nuclear pyknosis) preferentially affecting the paramedian areas bordering on the roof and floor of the third ventricle, particularly the preoptic and arcuate nuclei of the hypothalamus and neurons of the median eminence. Long-range effects, in mice given daily sc injections for the 10 days after birth (2·2–4·2 g/kg body weight) and observed to 9 months of age, included skeletal stunting, marked obesity with increased body weight, lethargy, poor pelage and female sterility. It was postulated that these findings comprised a "complex endocrine disturbance" related to the neonatal disruption of neuronal development of "regions of the brain thought to function as neuroendocrine regulatory centers".

The report also raised a question of a "risk to the developing human nervous system by maternal use of MSG during pregnancy". In a subsequent paper (Olney & Sharpe, 1969), the risk consideration was broadened to include human infants fed glutamate-enriched diets. To support such concern, the paper presented a description of acute neuronal necrosis in the periventricular-arcuate region of the hypothalamus of an infant rhesus monkey killed 3 hr after a single sc injection of 2·7 g MSG/kg given 8 hr after birth.

During this time the present study was planned and work was initiated. It was intended to provide information on several points, namely the question of whether the neuronal effects of single doses of MSG are reproducible, the influence of the route of administration (intragastric (ig) versus sc), the possible role of the degree of ionization of the material or

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the concentration of the sodium ion, possible species differences in the neuropathological effect of MSG and the influence of the age of the animal. In the latter connexion, the neonatal period (about 3 days after birth) was compared with the period when the lactating animal no longer depends completely on maternal milk and begins to take solid nourishment, a period about 12 days after birth for mice and rats and 35 days after birth for dogs. The MSG dose level used in the present study, 1 g/kg body weight, was high not only in relation to any conceivable intake by the human adult but also in relation to the 0·1–0·6 g MSG that might have been present in 4·5 ounces of baby foods formulated before the discontinuance of glutamate addition to baby foods by producers. It was considered reasonable therefore to focus on the 1 g/kg dose level in the present multi-species experiment.

EXPERIMENTAL

Materials. To evaluate the role of both the sodium and glutamate moieties of MSG and study the possible influence of ionization, four materials were tested, namely MSG, sodium chloride (completely ionizable), sodium gluconate (incompletely ionizable) and potassium glutamate, together with distilled water as a control. Consideration was given to the use of molar or molal equivalence, but it was decided to administer all test materials as sterile 10% (w/v) aqueous solutions, with animals receiving a constant dosage of 10 ml/kg body weight (equivalent to 1 g/kg).

Animals. The animals used were C57BL/6J infant mice, obtained from inhouse breeding of parent mice purchased from a commercial supplier, infant rats of the FDRL strain, beagle pups from the FDRL breeding colony and infant cynomolgus monkeys.

Treatment schedules. Both ig and sc routes of administration were used for each age group and species. Animals of each of the four species were dosed at or close to 3 days of age, and, in a second series, at ages corresponding to the period of introduction of 'solid' foods into the diet, as described above. A uniform dose level of 1 g/kg was used for all animals and test materials, except in the case of some monkeys, as indicated below.

Mice were divided into 20 groups of five, dosed either ig or sc at 3 or 12 days of age and killed 24 hr after dosage. Ten groups of eleven 3-day-old rats were also dosed by both routes, three rats from each group being sacrificed after 6 hr and eight after 24 hr. In addition, ten groups of five 12-day-old rats were similarly dosed and killed after 24 hr.

Three-day-old beagle pups were dosed by both routes in groups of six, one animal from each group being killed after 3 hr, two after 24 hr and three after 52 wk. The last three were observed daily and weighed monthly and at termination they were autopsied, the organs were weighed and femur length was measured (as an index of skeletal growth). Thirty additional dogs were treated (by sc and ig routes) at 35 days of age, one in each group of three being killed after 3 hr and two after 24 hr. Five additional groups of three 35-day-old pups were given gradually increasing ig doses (2·2–4·4 g/kg) over a 10-day period, according to the protocol of Potts, Modrell & Kingsbury (1960). Their progress was followed for 48 wk, at which time electroretinograms were recorded and examined. They were then killed and autopsied and organ weights and femur lengths were recorded. Extensive microscopic examinations were made of the brain, eyes and pituitary.

Thirty-two 3- or 4-day-old cynomolgus monkeys were studied. Doses of all the test compounds were administered at the 1 g/kg level both ig and sc in the form of 10% solutions and, in addition, two monkeys were given orally 4 g sodium chloride/kg as a 20% aqueous solution and five received 4 g MSG/kg orally. Of the latter group, one received the dose

in baby food and was killed 3 hr later, and the others were dosed with a 20% aqueous solution, two being killed at 3 hr and one each at 6 and 24 hr. The sodium chloride-treated monkeys were killed 3 and 24 hr after dosing.

Histopathology. Initially, rodents were anaesthetized with ether, the head was sagitally bisected and needle biopsies of the right ventral hypothalamus were taken immediately and fixed in 3% glutaraldehyde in phosphate buffer. Both halves of the brain were fixed in 10% neutral buffered formalin. All the mice and some rats and dogs were treated in this way. Subsequently the animals were anaesthetized (with ether for rats, Surital for dogs and Sernylan for monkeys) and cannulated into the ascending aorta through the left ventricle, and the brain was fixed by perfusion with 3% glutaraldehyde in phosphate buffer following a brief perfusion with heparinized saline or phosphate buffer. The glutaraldehyde perfusion time was about 10 min for rats and 15–20 min for dogs and monkeys. The fixed brain was removed and sectioned transversely through or near the pituitary stalk, and a 1-mm transverse slice just caudal to the stalk was removed, additionally fixed in glutaraldehyde and then stored in cold phosphate buffer for possible electron microscopic examination. The brain was stored in 10% neutral buffered formalin prior to further processing.

Gross examination of other organs (and storage of organs or samples in formalin) was carried out in the monkeys and in the dogs, other than those dosed when 3 days old and killed after 3 or 24 hr.

Examinations by light microscopy were made of 6-μm sections of paraffin-embedded tissue stained with haematoxylin and eosin. For some of the earlier studies, the left half of the brain of the mice and rats was sectioned sagitally. This was soon replaced by transverse or coronal sectioning for all other animals, and later coronal sections were prepared from the right half of the brain of many of the rodents studied earlier. Spaced sequential sections were taken throughout the hypothalamus, spanning the region from the preoptic level to the interpeduncular zone or premammillary level. The average number of sections examined for each animal was 30 for mice and rats, 20 for dogs and 20–30 for monkeys. In addition, light-microscopic examination was carried out on sections of certain endocrine organs from the 30 dogs dosed once at 3 days of age and followed for 52 wk.

Nearly all the slides were examined by two pathologists, one of whom read them blind.

RESULTS

In essentially every instance, microscopic examination of brain sections of animals aged 3–5 days showed occasional small neurons with nuclear pyknosis and a more frequent, but still sparse, scattering of small cells showing nuclear pyknosis or karyorrhexis with cytoplasmic eosinophilia and without any glial or inflammatory reaction. This kind of cell was widely distributed in the brain but was sometimes a little easier to find (presumably being more numerous) in the periventricular-arcuate region of the hypothalamus. However, these cells exhibited no apparent swelling (typically they are smaller than most of their neighbouring cells) and they were seen in the brains of animals of all groups.

Neuronal necrosis of the hypothalamic arcuate nuclei, as described and illustrated by Olney (1969), was not identified in any animal of any group, notwithstanding the variations in techniques of autopsy, fixation and sectioning. Only in one animal, a rat, dosed with 1 g MSG/kg at 3 days of age and killed 24 hr later, was an area seen that closely

resembled the lesion described by Olney (1969). In this animal, an area in the median eminence contained cells showing slight nuclear pyknosis and prominent vacuolation. However, most of the vacuolation appeared to be intercellular and was possibly an artefact, and the area was not believed to be a lesion attributable to the effect of MSG.

Particular attention was paid to the brain sections of the five monkeys dosed with 4 g MSG/kg and re-examination of the sections from most of the animals dosed with MSG or potassium glutamate revealed no changes with the reported features of MSG-induced necrosis, or even showing any difference from the other groups.

Table 1. *Body weights of dogs maintained to maturity following a single 1 g kg dose of MSG or other test material at 3 days of age*

Test compound	Dosage route	Dog no. and sex	Body weight (kg) at wk					
			0*	4	12	24	36	52
MSG	Oral	3201 F	0·47	1·21	4·6	8·6	10·0	10·2
		3202 F	0·36	1·10	2·9	6·6	7·6	7·7
		3203 M	0·44	1·21	5·4	9·5	11·1	11·1
		Mean...	0·42	1·17	4·3	8·2	9·6	9·7
	Sc	3207 F	0·51	1·22	3·9	7·4	8·3	8·7
		3208 M	0·54	1·27	6·4	10·2	16·1	16·6
		3209 M	0·60	1·35	5·7	9·2	13·5	15·7
		Mean...	0·55	1·28	5·3	8·9	12·6	13·7
NaCl	Oral	3213 F	0·56	1·24	3·9	7·8	8·7	11·3
		3214 F	0·33	1·05	3·0	7·5	8·5	9·0
		3215 F	0·48	1·20	5·1	8·7	10·6	11·0
		Mean...	0·46	1·16	4·0	8·0	9·3	10·4
	Sc	3219 F	0·52	1·20	3·6	8·0	8·7	9·7
		3220 M	0·42	1·16	5·6	8·6	11·6	12·0
		3221 M	0·61	1·32	6·1	9·3	12·5	11·7
		Mean...	0·51	1·23	5·1	8·6	10·9	11·1
Na gluc	Oral	3225 F	0·54	1·25	3·6	7·9	8·8	10·3
		3226 F	0·27	1·09	3·8	7·6	8·2	8·7
		3227 M	0·46	1·29	6·1	10·4	15·3	15·1
		Mean...	0·42	1·21	4·5	8·6	10·8	11·4
	Sc	3231 F	0·48	1·10	3·9	7·5	8·9	9·1
		3232 F	0·46	1·12	4·8	9·0	11·3	12·0
		3233 M	0·62	1·30	6·2	9·4	15·4	16·4
		Mean...	0·52	1·17	5·0	8·6	11·9	12·5
MKG	Oral	3237 F	0·37	1·10	4·0	7·9	7·9	7·7
		3238 M	0·48	1·23	4·5	9·2	12·0	11·6
		3239 F	0·61	1·36	4·8	9·6	12·0	12·4
		Mean...	0·48	1·23	4·4	8·9	10·6	10·6
	Sc	3243 F	0·56	1·31	5·1	9·5	11·3	11·3
		3244 F	0·40	1·10	3·7	7·5	9·9	9·7
		3245 F	0·54	1·24	4·8	8·6	9·7	9·4
		Mean...	0·50	1·22	4·5	8·5	10·3	10·1
Water	Oral	3249 F	0·55	1·15	3·5	7·6	8·9	9·6
		3250 F	0·25	1·12	3·5	7·2	8·2	8·7
		3251 F	0·35	1·11	4·8	8·5	8·8	8·6
		Mean...	0·38	1·13	3·9	7·8	8·6	9·0
	Sc	3255 M	0·55	1·28	5·6	9·4	14·3	15·1
		3256 M	0·53	1·21	4·9	8·4†	—	—
		3257 M	0·37	1·15	4·9	8·8	12·8	14·1
		Mean...	0·48	1·21	5·1	8·9	13·6	14·6

MSG = Monosodium glutamate Na gluc = Sodium gluconate MKG = Monopotassium glutamate
*Initial body weight.
†Death ensued before the next weighing.

Microscopic examination of the eyes of these animals, and scrutiny of the electroretinograms of neonatal dogs dosed serially according to the procedure of Potts *et al.* (1960) and Cohen (1967), failed to reveal any effect of MSG or any significant difference between the groups.

Gross and microscopic examination was carried out on the pituitary gland, ovaries, uterus and mammary glands of the 30 dogs dosed once at 3 days of age and observed for 52 wk. No abnormalities or differences between groups were found. Body weights, recorded monthly during the 52 wk these animals were followed, showed no evidence of an effect of any treatment (Table 1).

In the additional group of 15 dogs dosed for 10 days, starting when they were 35 days old, and autopsied at 48 wk, no unusual or treatment-related changes were found in the gross examination or weights of organs (gonads, liver, spleen, kidneys, adrenals, thyroid, pituitary, heart and brain), in femur lengths, in electroretinograms or in the microscopic examination of the brain, eyes and pituitary.

Slight regurgitation of mucus was noted in one of the sodium chloride-treated monkeys and at autopsy (24 hr after dosing) the stomach mucosa appeared reddened. The other member of this pair was sacrificed 3 hr after dosing. The stomach and upper small intestine contained blood and the mucosa appeared haemorrhagic. No such findings were seen in the animals on MSG.

DISCUSSION

During the past few decades, MSG has been the subject of a number of toxicological and pharmacological investigations in several animal species, including man. Retinotoxic properties were reported by Lucas & Newhouse (1957), who described degeneration of the ganglion-cell layer and failure of formation of the inner nuclear layer of the retina. Inhibition of formation of the inner retinal layers was confirmed by Potts *et al.* (1960), following ip dosing of MSG to infant mice at daily increasing levels for 2–17 days. These authors proposed that, in the mouse, MSG repressed an essential enzyme needed for the development of the inner retinal layers. Cohen (1967) reported the destruction of axons in the optic nerves of 2-month-old mice given MSG as neonates (2·2–4·2 g/kg on days 1–10 after birth).

The present investigation was prompted by the report that sc administration of high doses of MSG (0·5–4 g/kg) exerted a neurotoxic effect on several regions of the brain of neonatal rodents (Olney, 1969) which, as previously mentioned, was interpreted as suggesting a neurological hazard to the developing human foetus "by maternal use of MSG during pregnancy". The relevance of the original Olney (1969) study to the safety of MSG as used in foods was questioned (Blood, Oser & White, 1969) on the grounds of the age of the test animals, the routes of administration, the magnitude of the doses and the lack of adequate controls. Several additional correspondents (Lowe, 1970; McLaughlan, Noel, Botting & Knipfel, 1970; Zavon, 1970) questioned the work of Olney (1969) because (a) data were lacking on the response of control mice to the solvent alone or to equivalent amounts of sodium chloride or the salts of other amino acids; (b) no blood-level data were gathered; (c) only one primate was used; and (d) the levels of MSG used in the neurotoxicity studies seemed excessively high in relation to potential human ingestion. Olney & Sharpe (1969, 1970) later supplied some of the information lacking in the original report, as well as additional data which supported their conclusion about the effect of high doses of MSG in the infant animal.

Adamo & Ratner (1970) studied both acute and long-range effects in rats given a single sc injection of 4 mg MSG/kg when 3 or 4 days old and were unable to find any evidence of an adverse effect of glutamate on neural morphology or the reproductive system.

Arees & Mayer (1970) reported that approximately 95% of their MSG-treated mice, given a single sc or ip injection (at a dosage of 2 or 4 g/kg for infant mice and 6–10 g/kg for adult mice) and killed 3–72 hr later, showed lesions in the arcuate nuclei of the hypothalamus. However, the lesions were smaller than those reported by Olney (1969) and consisted primarily of degenerating microglial cells, the perikarya of neurons being unaffected.

Olney & Ho (1970) reported brain damage in 10–12-day-old mice killed 3–6 hr after receiving a single oral dose of MSG (dosage 0·5–2 g/kg) or monosodium aspartate (dosage 1 g/kg). The arcuate nucleus of the hypothalamus showed well-demarcated lesions, in which the number of necrotic neurons in each cross-section of the arcuate nucleus was dose-related. Neuronal cytopathology was not found in controls, which included some animals treated with "massive" doses of sodium chloride or "large amounts" of sodium bisulphite or glutarate.

Reynolds, Lemkey-Johnston, Filer & Pitkin (1971) found hypothalamic lesions (neuronal necrosis) in the newborn mouse following oral administration of MSG, but in their study of infant monkeys no hypothalamic changes could be found in animals sacrificed 6 hr after ig administration of 1–4 g/kg. Abraham, Dougherty, Golberg & Coulston (1971) distinguished two types of lesions in the arcuate nuclei of mice given MSG, ig administration involving glial cells and sc administration involving neuronal cells. The incidence of lesions was higher at a dosage of 4 g/kg than at 1 g/kg, and higher with sc than ig administration. In a similar examination in which the hypothalamus of four infant monkeys given 4 g MSG/kg (two orally and two sc) was compared with that in control infant monkeys, no effect was found. The authors suggested that the species difference in susceptibility to large doses of MSG may relate to the disparate permeability of the blood–brain barrier in neonatal animals consequent upon different degrees of myelination of the central nervous system. The rate of intestinal absorption was suggested as a limiting factor in the hypothalamic effect in mice.

In the studies here reported, involving the ig or sc administration of single 1 g/kg doses of MSG to rats and mice 3 or 12 days of age or to dogs 3 or 35 days of age, and killed 3 hr (dogs), 6 hr (rats) or 24 hr (rats, mice and dogs) after dosage, no hypothalamic pathology was observed. Single 4 g/kg ig doses given to five infant monkeys were likewise negative. Growth, appearance and behaviour appeared normal and gross and microscopic examination at autopsy revealed no abnormalities in the brain, eyes, pituitary, ovaries and uterus of dogs given a single dose at 3 days of age and observed for 52 wk. No evidence of pathological change was found in electroretinograms or in the eyes or brain of dogs given increasing daily doses for 10 days from 35 days of age and followed for 48 wk.

Several possible reasons may be suggested for this failure to observe neurotoxic effects with MSG. The hypothalamic lesion described by Olney (1969) may be so slight as to be difficult to find by any technique short of examination of serial sections of the entire ventral hypothalmus. This study was not designed to detect and quantitate lesions of such minute degree. However, if even very small lesions had been present in these animals, it seems improbable that the examination of periodic or spaced sections would miss them every time. The thickness of light microscopic sections and the stain used may be important in the optimal visualization of the lesion. This study used 6-μm sections stained with

haematoxylin and eosin, whereas Olney & Sharpe (1969) used 1-μm sections stained by the Richardson method (Richardson, Jarett & Finke, 1960). Considering the light microscopic features of MSG neuropathology (swollen cell, vacuolated cytoplasm and nuclear pyknosis), it seems to us unlikely that these effects would be shown by only one of these stains.

It should be noted that in a more recent report (Olney, Sharpe & Feigin, 1972) the single 1-wk-old monkey dosed orally at 1 g/kg showed "tiny lesions" consisting of acutely necrotic neurons "almost exclusively in rostral subventricular nucleus". At doses of 2 or 4 g/kg, such lesion sites were more conspicuous and somewhat greater in number.

The histological technique for fixing tissues is of crucial importance in electron microscopy, but is not so critical in light microscopy, at least not for the features claimed to be induced by high doses of MSG. Some of the initial work here reported was done with ordinary formalin fixation; when the perfusion method was first used, the technique failed in occasional animals. Nevertheless variations in fixation procedures provided ample opportunity for revealing the presence of hypothalamic lesions, if in fact they were present. The possibility that inadequate fixation might somehow reverse the lesions so that necrotic cells would appear normal is hardly tenable.

Oral or ig dosage is subject to criticism on the grounds of possible loss of test material by regurgitation or vomiting (either from an excessive dosage volume or an emetic action of the material). The absence of MSG effects in this study cannot be due to this factor, however, because lesions should then have been found in animals dosed sc.

No adverse effect on neurological or hepatic function was observed by Bazzano, D'Elia & Olsen (1970) in human adult males receiving as much as 137 g glutamic acid daily for periods ranging from 14 to 41 days, nor in gerbils fed diets containing glutamate at a level equivalent to 30 g/kg body weight/day. These authors, like others, suggest that the central question is how much of the fed or injected MSG actually crosses the blood–brain barrier in the neonatal animal and whether the rate of development of the blood–brain barrier in rodents or primates is comparable to that in the human infant. There is no doubt that passage across the barrier is a function of the blood level and, in turn, of dose and concentration.

Though the existence of a blood–brain barrier has been questioned, the evidence concerning a wide series of active agents shows that, with maturation, there is a "sparing" action (i.e. a lower central sensitivity to toxicants) indicative of the development of such a barrier. Its specific time of appearance is largely determined by functional tests, but it has been shown to be present when the animal begins to ingest the 'solid' food offered in the maternal diet.

Several reports of induced hypothalamic lesions involved a 4 g/kg daily dose, whereas 1 g/kg was used in most of these studies. The higher dose would be equivalent to about 15 g for a newborn human infant. The administration of massive dosages to 1–3-day-old rats places a substantial toxic stress on the neonatal organism when considered in the light of the fact that metabolic enzyme systems are still in the stage of development during the period of lactation.

The present study fully supports the conclusion of the Food Protection Committee (1970) that the risk associated with the use of MSG in infant food is extremely small and that, except for persons individually sensitive to MSG, foods containing the flavour enhancer present no hazard for older children and adults.

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SHORT-TERM TOXICITY OF DIMETHYL SULPHIDE IN THE RAT

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Abstract—Groups of 15 male and 15 female rats were given doses of 0 (control), 2·5, 25 or 250 mg dimethyl sulphide/kg/day for 14 wk. No effects on the rate of body-weight gain, intake of food and water, results of haematological examinations, serum-enzyme levels, urinary cell excretion, renal concentration tests, organ weights or histopathological examinations were attributable to the treatment. A no-untoward-effect level of 250 mg/kg body weight/day was therefore established.

INTRODUCTION

Dimethyl sulphide (DMS; methyl sulphide; methylthiomethane; CH_3-S-CH_3) is currently used in the UK as a constituent of artificial flavouring agents. It is employed in ice cream, blancmanges and jellies and sugar and flour confectionery, as well as in soft drinks. There are, at present, no specific regulations governing the use of flavourings in the UK. The Food Standards Committee (1965), in its Report on Flavouring Agents, suggested 17 flavourings which should not be used in foods, but DMS was not one of these. It is classed as temporarily admissible by the EEC, but acute, short-term and metabolic studies are required by 1975 if the permission for its continued use is to be substantiated. The Council of Europe (1972) suggested a limit of 1·5 ppm in foodstuffs.

DMS is included (as FEMA no. 2746) in the list of flavourings "generally recognized as safe" by the Expert Panel of the Flavour and Extract Manufacturers' Association and its use is permitted under Sec. 121.1164 of the Code of Federal Regulations of the Food and Drug Administration.

The levels of DMS present in various beers have been determined by Sinclair, Hall, Burns & Hayes (1970). It occurs naturally in British ales and lagers, at concentrations found to be less than 30 μg/litre. This may be compared with the range of 120–140 μg/litre found by Drews, Specht & Kuhl (1966) in Pilsner-type beers. Patton, Forss & Day (1956) have reported that it is one of the natural flavour constituents of cow's milk. Its natural occurrence has also been noted by Challenger (1951), who reported that many living systems produce DMS and that prominent among these are marine algae. Lovelock, Maggs & Ramussen (1972) have suggested that DMS is the natural sulphur compound that fits the role originally assigned to hydrogen sulphide, namely that of transferring sulphur from the sea through the air, as a gas, to land surfaces.

Concerning the metabolism of DMS, Maw (1953) found that it was easily detectable in the exhaled air of rats after administration in the diet or by injection. This finding after dietary administration may be criticized on the grounds of its high volatility, but, when administered by injection as a solution in arachis oil, DMS was clearly detectable in the breath of rats within 1 hr of dosing. Maw (1953) also observed that there was no increase

in the output of sulphate in the urine of these rats. Williams (1959) suggested that a possible metabolic fate of DMS would be its oxidation to dimethylsulphone. Williams, Burstein & Layne (1966) stated that the demethylation of DMS did not seem to be an important reaction in the rabbit. They found dimethylsulphoxide and dimethylsulphone in the urine of their animals and suggested that these substances were the major metabolites. They considered it probable that the remainder of the dose was expired unchanged by way of the lungs and that some was excreted in the faeces, although the faeces were not examined. In contrast, DMS has been shown to be a minor metabolite of dimethylsulphoxide in several species. DiStefano & Borgstedt (1964) identified DMS in the expired air from cats within 1 min of the iv injection of dimethylsulphoxide.

There are no reports of studies of the toxicity of DMS and so the short-term study in rats described in this paper was undertaken as part of the BIBRA safety evaluation programme.

EXPERIMENTAL

Materials. DMS was obtained from the Sigma Chemical Co. Ltd., London, and complied with the following specification: Purity, min. 99%; specific gravity (at 20°C), 0·845–0·852; refractive index (at 20°C), 1·432–1·437; m.p., −83·2°C; b.p., 37·2°C. The corn oil used as the diluent was supplied by J. Sainsbury Ltd., London.

Animals and diets. Weaning SPF rats of the Wistar strain, obtained from A. Tuck & Son, Rayleigh, Essex, were given ground Spillers' Laboratory Small Animal Diet and water *ad lib.* They were housed five in a cage, in an animal room maintained at 21 ± 1°C with a relative humidity of 50–60%.

Experimental design and conduct. Groups of 15 male rats (body weight 75–90 g) and 15 females (body weight 70–85 g) were given DMS by daily oral intubation (7 days/wk) at dose levels of 0 (control), 2·5, 25 or 250 mg/kg/day for 14 wk. In addition, groups of five rats of each sex and of similar body weight were given daily doses of 0, 25 or 250 mg DMS/kg for 2 or 6 wk. The DMS was administered as a solution in corn oil and the concentrations were adjusted so that all rats received a dosage volume of 5 ml/kg/day.

The animals were weighed on day 0 and then weekly throughout the test. Their consumption of food and water was measured over a 24-hr period preceding the day of weighing. During wk 2, 6 and 14 of treatment, urine was collected from the rats and examined for appearance, microscopic constituents and content of glucose, ketones, bile salts and blood. A concentration and dilution test was carried out on the same animals, involving the measurement of the specific gravity and volume of urine produced in a 6-hr period of water deprivation and in a 2-hr period following a water load of 25 ml/kg. In addition, in the groups examined at wk 6 and 14 the same measurements were made on the urine produced between 16 and 20 hr after the water load.

At the end of the appropriate period of dosing, the rats were killed by exsanguination under anaesthesia with pentobarbitone sodium following a 24-hr period without food. Blood was taken from the aorta for haematological studies and serum analyses. At autopsy any gross abnormalities were noted and the brain, pituitary, thyroid, heart, liver, stomach, small intestine, caecum, spleen, kidneys, adrenals and gonads were weighed. Samples of these organs and of salivary gland, trachea, oesophagus, colon, rectum, lymph nodes, lung, aorta, pancreas, urinary bladder, uterus and skeletal muscle were preserved in 10% buffered formalin. Paraffin-wax sections of these tissues were stained with haematoxylin and

eosin for microscopic examination. This examination was performed on the tissues from the rats given 250 mg DMS/kg/day for 14 wk and from half of the control rats. In addition, the tissues from animals in which abnormalities were suspected at autopsy were examined.

Blood taken at autopsy was examined for haemoglobin content, packed cell volume and counts of erythrocytes and total leucocytes. Differential leucocyte counts were made on all rats from the control group and those given 250 mg DMS/kg/day. Reticulocyte counts were made on the blood of the same animals at wk 6 and 14. Serum from all rats was analysed for the activities of glutamic–oxalacetic and glutamic–pyruvic transaminases and lactic dehydrogenase.

RESULTS

All the rats appeared to be in good health throughout the study and no abnormalities in behaviour were observed. There were neither significant differences between the control and test groups of animals nor dose-related variations between the groups with respect to their food and water intake or in their rate of body-weight gain (Table 1). Similarly there were no significant differences between the treated and control groups in the results of the haematological examinations (Table 2), serum enzyme levels or urinary cell excretion (Table 3). There were no abnormal urinary constituents in any of the animals. Although there were scattered changes in the volume and specific gravity of the urine, these were neither dose-related nor seen consistently in both sexes (Table 3).

No statistically significant differences in organ weights were observed after treatment for 2 wk but, when expressed relative to body weight, the brain weight in the group of female rats given 250 mg/kg/day showed a statistically significant increase at this stage compared with those of the control animals. There was no comparable increase in the brain weights of the male rats. At wk 6 there was a higher splenic weight in the male rats given 25 mg/kg/day and a lower heart weight in the females given 250 mg/kg/day. However, when these weights were expressed relative to body weight, the differences were no longer statistically significant. A reduction in the relative stomach weight in male rats given 25 mg/kg/day was statistically significant. At wk 14 the weight of the small intestine of the male rats was greater than that of the controls at all three dose levels (Table 4). However, when this weight was expressed relative to body weight, the difference was no longer statistically significant in the rats given 2·5 mg/kg/day (Table 4). The female rats dosed with 250 mg/kg/day had a lower thyroid-gland weight than controls, the decrease being statistically significant ($P < 0{\cdot}01$) both for the absolute and relative weight. Conversely, the corresponding male rats had heavier thyroid glands, although this was statistically significant only in respect of the relative organ weight.

At autopsy, occasional pitting of the cortex of the kidneys and pallor of the liver were seen. Histopathological examination revealed some degree of fatty degeneration of the liver cells and some chronic inflammation of both the lungs and kidneys. These changes were comparable in incidence and severity in the treated and control animals.

DISCUSSION

Some of the organ weights were found at autopsy to differ significantly from the values for the control animals. These differences were random, were not dose-related, and were not matched by the results obtained in the comparable group of rats of the opposite sex. They may therefore be assumed to have occurred by chance. The decrease in the mean

Table 1. *Mean body weight, food consumption and water consumption of rats dosed with 0–250 mg DMS/kg/day for 90 days*

Dose level (mg/kg/day)	Body weight (g) at day 0*	34	62	90	Weight gain at day 90 (g)	Food consumption (g/rat/day) at day 0*	34	68	90	Mean consumption (g/rat/day)	Water consumption (ml/rat/day) at day 0*	34	62	90	Mean consumption (ml/rat/day)
Males															
0	83	277	355	397	314	11·4	18·3	16·3	18·7	17·6	16·2	24·5	24·5	30·0	25·7
2·5	84	290	378	425	341	11·9	18·6	16·7	17·5	17·6	15·9	22·9	22·6	22·8	24·0
25·0	82	283	361	406	324	11·3	18·3	16·7	16·9	17·8	15·3	25·1	22·7	24·5	23·9
250·0	84	286	364	406	322	12·1	18·9	17·9	17·8	18·2	17·2	26·6	25·1	27·7	26·9
Females															
0	78	191	222	237	159	10·7	14·3	13·9	16·4	14·5	17·7	33·1	25·0	29·9	25·2
2·5	79	191	221	236	157	10·7	14·7	15·1	18·3	14·8	16·5	23·1	28·9	26·3	24·7
25·0	79	193	233	245	166	11·4	14·5	15·2	15·7	15·3	15·7	25·7	32·5	32·9	27·9
250·0	77	188	225	235	158	10·2	14·0	14·6	15·0	15·2	15·9	23·5	31·4	32·7	28·9

*First day of treatment.

Body weights are means for 15 animals. Values for food and water consumption are the means for three cages of five animals. Although growth and water consumption were recorded weekly, only values at monthly intervals are included in the Table. Values do not differ significantly from those of controls at $P = 0{\cdot}05$ (Student's t test).

Table 2. *Haematological values in rats dosed with 0–250 mg DMS/kg/day for 14 wk*

Dose level (mg/kg/day)	Hb (g/100 ml)	PCV (%)	RBC (10^6 mm^3)	Retics (% of RBC)	Leucocytes: Total (10^3/mm^3)	Leucocytes: Differential (%) N	E	L	M
Males									
0	14·0	44	5·83	0·9	4·99	17	1	81	1
2·5	14·3	44	6·03	—	5·03	—	—	—	—
25	14·5	44	6·02	—	4·12	—	—	—	—
250	14·4	44	5·98	0·7	5·03	18	1	80	1
Females									
0	14·2	42	6·14	1·0	3·76	16	1	81	2
2·5	13·9	42	5·92	—	3·88	—	—	—	—
25	13·9	42	5·84	—	3·63	—	—	—	—
250	13·9	42	5·68	0·9	3·74	15	1	82	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 and the results for treated animals did not differ significantly from those of the control animals (Student's *t* test) at $P = 0{\cdot}05$. Determinations at wk 2 and 6 were similarly free of any positive effect. Basophils did not account for more than 0·5% of the leucocytes in any group.

Table 3. *Mean values of renal concentration/dilution tests and urinary cell excretion of rats dosed with 0–250 mg DMS/kg/day for 6 or 14 wk*

Sex and dose level (mg/kg/day)	Cells (10^3/hr)	Concentration test: Specific gravity 0–6 hr	Specific gravity 16–20 hr	Volume (ml) 0–6 hr	Volume (ml) 16–20 hr	Dilution test (2 hr): Specific gravity	Volume (ml)
Wk 6							
Male							
0	2·4	1·055	1·085	3·4	0·3	1·010	5·8
25	4·8	1·076	1·087	2·2	0·2	1·006	7·1
250	4·5	1·060	1·084	2·0*	0·5	1·007	6·4
Female							
0	4·1	1·053	1·073	2·0	0·1	1·006	3·5
25	3·7	1·065	1·073	2·3	0·4*	1·008	4·5
250	3·0	1·063	1·076	3·0	0·1	1·004	3·2
Wk 14							
Male							
0	5·2	1·068	1·079	2·1	0·5	1·004	8·7
2·5	7·4	1·067	1·081	2·4	0·5	1·005	8·8
25	7·1	1·068	1·091**	1·6	0·4	1·005	7·4
250	5·5	1·060	1·084	2·3	0·3	1·004	7·3
Female							
0	5·7	1·059	1·093	1·4	0·3	1·004	3·8
2·5	6·2	1·052	1·089	1·8	0·3	1·004	4·3
25	6·4	1·061	1·091	1·4	0·2	1·004	3·7
250	5·7	1·041**	1·086	1·0	0·3	1·004	3·8

Results are means for groups of five rats at wk 6 and of 15 rats at wk 14. Those marked with asterisks differ significantly (Student's *t* test) from those of the appropriate controls: $*P < 0{\cdot}05$; $**P < 0{\cdot}01$.

No differences between treated and control animals were found at wk 2, and tests for glucose, bile salts, blood and ketones were negative throughout.

Table 4. *Mean organ weights and relative organ weights of rats dosed with 0–250 mg DMS/kg/day for 14 wk*

Sex and dose level (mg/kg/day)	Weights of												Terminal body weight (g)
	Brain	Heart	Liver	Spleen	Kidneys	Stomach	Small intestine	Caecum	Adrenals†	Gonads‡	Pituitary†	Thyroid†	
Organ weight (g)													
Male													
0	1·85	1·02	10·70	0·68	2·17	1·61	8·53	1·01	53·6	3·46	9·09	16·7	388
2·5	1·85	1·04	11·43	0·70	2·21	1·60	9·34*	1·02	56·3	3·40	9·25	18·2	413
25	1·83	1·00	10·85	0·72	2·18	1·60	9·42*	1·00	52·6	3·49	8·68	18·7	393
250	1·86	1·01	11·21	0·68	2·17	1·63	9·54*	1·04	59·1	3·58	9·00	19·7	396
Female													
0	1·76	0·72	6·38	0·54	1·38	1·28	7·03	0·81	68·4	109	11·61	15·6	229
2·5	1·78	0·71	6·43	0·59	1·39	1·30	7·00	0·79	68·3	113	11·81	14·1	229
25	1·80	0·76	6·69	0·61	1·47	1·32	7·54	0·79	76·4	110	12·93	13·7	235
250	1·73	0·71	6·04	0·56	1·43	1·31	6·72	0·77	65·4	108	11·46	12·0**	228
Relative organ weight (g/100 g body weight)													
Male													
0	0·48	0·26	2·74	0·16	0·56	0·42	2·21	0·26	13·7	0·89	2·36	4·31	
2·5	0·45	0·25	2·76	0·17	0·54	0·39	2·27	0·25	13·8	0·83	2·24	4·42	
25	0·47	0·25	2·76	0·18	0·56	0·41	2·40*	0·25	13·4	0·89	2·22	4·78	
250	0·48	0·26	2·82	0·17	0·55	0·41	2·41*	0·26	15·1	0·92	2·25	5·12*	
Female													
0	0·78	0·32	2·79	0·24	0·61	0·56	3·00	0·35	30·2	48·3	5·08	6·82	
2·5	0·78	0·31	2·80	0·26	0·61	0·57	3·07	0·35	29·8	49·3	5·15	6·22	
25	0·77	0·32	2·85	0·26	0·63	0·56	3·23	0·33	32·8	46·6	5·49	5·91	
250	0·76	0·31	2·66	0·24	0·63	0·57	2·95	0·34	28·9	47·4	5·05	5·25**	

†Weights and relative weights of these organs are expressed in mg and mg/100 g body weight, respectively.
‡Weights and relative weights of the female gonads are expressed in mg and mg/100 g body weight, respectively.
Values are means for groups of 15 animals and those marked with asterisks differ significantly (Student's *t* test) from those of the controls: $*P < 0·05$; $**P < 0·01$.

weight of the heart in the female rats at the high dose level (250 mg/kg/day) at wk 6 can be explained by the reduced body weight of this group of animals. This is confirmed by the normal value obtained when the heart weight was expressed relative to body weight. The changes in the weights of the thyroid glands were significant at the top level at wk 14, but the changes were in opposite directions in the two sexes and are therefore thought to be of no significance.

The weights of the small intestine of all male groups given DMS for 14 wk were greater than those of the controls and the differences were statistically significant, although there was no similar finding in the male groups at earlier examinations or in the female groups at any time. There was only a 2% difference between the weight of the lowest and highest doses, despite a 100-fold increment in dose level. This indicates the absence of a dose-response relationship. This was confirmed by an F test involving all the treated rats, where the value of F was not significant. This shows that there were no differences between the values in these groups. On the other hand, the value of F was significant ($P < 0{\cdot}05$) when the control values were included, showing that any differences were between the controls and the treated animals and not between the different levels of treatment. Five other studies in these laboratories using the same experimental conditions, including dosing with oil, have shown that the mean control weight of the small intestine varies from 7·91–10·4 g. The overall mean value from these five studies was 8·92 g, a value higher than those of the present controls and not differing significantly from that for the animals given DMS. Moreover the scatter of this particular group was low, with a coefficient of variation of 9·9% compared with up to 19·8% for the other five studies. Groups with such a low variation would tend to show statistically significant differences, particularly at the $P < 0{\cdot}05$ level, without this representing a true effect of treatment. It is concluded that these increased intestinal weights did not represent an effect of DMS, but only reflected a control group with intestinal weights that were lower and showed a smaller scatter than that normally found. In addition, no histopathological changes were detected in the intestine and it is improbable that absorption from the small intestine was impaired, since there was no adverse effect on the body weights of the rats. Because of these considerations it is unlikely that the heavier intestines found at wk 14 indicated a true effect of DMS and it is probable that the figures for the intestinal weights of the control male animals are atypical.

In the urinary concentration test there were two variations in the volume of urine produced and two in the specific gravity of the urine. Although these results were statistically different from the values of the control animals, the changes consisted of both elevations and reductions and showed no consistent time- or dose-response relationship. Therefore these are not considered to be related to the administration of the DMS.

Since no effects attributable to treatment were seen at any dose level, the no-untoward-effect level for DMS in this study was taken as 250 mg/kg/day, which is equivalent to an intake of 17·5 g/day by a 70-kg adult. From the data supplied by seven of the leading flavouring manufacturers, it may be calculated that the maximum likely intake of DMS is probably about 1·7 mg/day. Thus the no-untoward-effect level in this study is about 10,000 times the maximum likely intake in man.

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STUDIES ON THE METABOLIC FATE OF ^{32}P-LABELLED EMULSIFIER YN IN THE MOUSE, GUINEA-PIG AND FERRET

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Abstract—The metabolic fate of Emulsifier YN (YN) was studied in the mouse, guinea-pig and ferret. Following a single oral dose of YN labelled in the phosphatidic acid component with ^{32}P, most of the radioactivity was excreted in the faeces within 48 hr. Urinary excretion accounted for 10–20% of the administered label and persisted at a low level for up to 10 days. Studies on *in vitro* hydrolysis by intestinal mucosa, on intestinal absorption and on whole-body autoradiography and a comparison of the excretion pattern for YN with that following a single dose of inorganic [^{32}P]phosphate suggested that YN was rapidly hydrolysed to inorganic phosphate and lipid in the intestinal tract and disposed of along normal physiological pathways.

INTRODUCTION

Emulsifier YN (subsequently referred to as YN) is used as an emulsifier and viscosity-reducing agent in the manufacture of chocolate. The material consists of triglycerides and a mixture of the ammonium salts of phosphatidic acids derived from partially hardened rapeseed oil. Six phosphatidic acids have been identified in the product and it is probable that the relative proportions of these vary with different batches. Inorganic phosphorus is also present in the final product as ammonium phosphate.

Feuer (1967) studied the metabolism of ^{32}P-labelled YN in the rat, and showed that 80% of the radioactivity was excreted in the faeces. From *in vitro* studies he concluded that the remainder was hydrolysed in the gastro-intestinal tract to inorganic phosphate and disposed of along physiological pathways. Brantom, Gaunt, Hardy, Grasso & Gangolli (1973) demonstrated a no-untoward-effect level of 6% for orally administered YN in the rat in a 2-yr feeding and reproduction study. While accepting the results of the rat studies, the Joint FAO/WHO Expert Committee on Food Additives (1970) recommended that the metabolism of this formulation should be investigated in several animal species.

The results reported in this paper extend the work on the metabolism of YN to the mouse, guinea-pig and ferret, to provide data for the further assessment of the safety of the formulation as a food additive.

EXPERIMENTAL

Materials. Non-labelled YN was prepared by Cadbury Bros., Bournville, Birmingham. [^{32}P]YN (2 mCi/g) was supplied by the Radiochemical Centre, Amersham, Bucks. Analysis by Cadbury Bros. showed that the material conformed to the standard specification for YN. Inorganic [^{32}P]phosphate, as ortho-phosphate solution (10 mCi/ml) was also

obtained from the Radiochemical Centre, Amersham. In all the experiments described, YN was administered to the animals or used as a substrate for *in vitro* studies in the form of an emulsion. The appropriate weight of [^{32}P]YN or unlabelled YN was dissolved in 2-methoxyethanol, diluted with 9 vols 0·9% w/v saline solution and sonicated (MSE 150 W Ultrasonic Disintegrator). The homogenous emulsion thus formed was stable at room temperature for at least 1 hr and was stored at 0°C.

Animals and diets. The studies were carried out on male guinea-pigs of the Dunkin/Hartley strain (250–300 g body weight), on male albino ferrets (*Putorius putorius*) obtained from the Wellcome Veterinary Research Laboratory, Tunbridge Wells, Kent, on female Wistar rats (180–220 g body weight) and on male mice of the LACA strain bred under specified-pathogen-free conditions. The mice used for the autoradiographic study were weanling animals (10–20 g body weight), while those used for the intestinal-absorption experiment were 12–14 wk old (35–40 g body weight).

The rats and mice were maintained on Spillers' Laboratory Small Animal Diet and water *ad lib.* and were kept at a temperature of 20 ± 2°C. Guinea-pigs were given Oxoid diet SG1 and vitamin C supplement in the drinking-water and ferrets were given Spratts ZF6 canned food and water *ad lib.* For the whole-animal metabolism studies the guinea-pigs were housed in all-glass metabolism cages (Jencon Ltd., Hemel Hempstead, Herts.) and the ferrets were kept in metal cages with mesh flooring and an inverted cone-shaped tray underneath. The tray was lined with polythene sheet, and at the apex of the cone a hole allowed the urine to run through into a collection vessel. Faeces were recovered from the cage floor and polythene sheet.

Metabolic studies in vivo. Three male guinea-pigs were given, by oral intubation, [^{32}P]YN emulsion (4 ml/kg, 40 μCi/kg) providing a dose level of 200 mg/kg, and urine and faeces were collected at 24, 48, 72, 96, 168 and 240 hr. The urine was centrifuged and aliquots of the clear supernatant were assayed for radioactivity. The white precipitate was dissolved in 2 N-HCl and aliquots were assayed for radioactivity. Faeces were dried *in vacuo* over phosphorus pentoxide overnight and a portion (400 mg) was combusted in air at 450° C. The residue was dissolved in conc. HCl (1 ml) and filtered to remove debris and aliquots were counted for radioactivity.

Three other male guinea-pigs were orally intubated with YN emulsion (4 ml/kg; 200 mg/kg) containing [^{32}P]phosphate (40 μCi/kg). Urine and faeces were collected at intervals of 24 hr for 10 days and the [^{32}P] content was determined as described above.

Two male ferrets were given, by oral intubation, [^{32}P]YN emulsion (7 ml/kg, 23 μCi/kg) providing a dose level of 90 mg/kg, and this was followed by collection of urine at 24, 48 and 72 hr and faeces at 24, 48, 72, 96, 120 and 144 hr. Aliquots of the urine were assayed for radioactivity. Faeces were treated as described for the guinea-pig, and total radioactivity was determined.

Autoradiography. Eight mice were given, by oral intubation, 0·2 ml [^{32}P]YN emulsion. The mice were starved overnight before being dosed and received approximately 10 mg YN (1 μCi/mg YN). Single animals were killed at 1, 2, 4, 8, 24, 72, 120 and 168 hr after dosing by exposure to diethyl ether vapour. They were immediately frozen by immersion in acetone-solid CO_2 and stored at −20°C for 6 hr for the acetone to evaporate. The mice were blocked in 1% carboxymethylcellulose and 100 μ sections of the whole body were prepared as described by Ullberg (1954). Sections containing samples of all tissues and organs were dried over silica gel at −20°C and placed in contact with X-ray film (Agfa Structurix DX5) at −20°C for 24 or 48 hr in the dark. After exposure the film was devel-

oped in Kodak DX80 developer and fixed in acid thiosulphate to demonstrate the areas that had been exposed to radiation from the sections.

Intestinal absorption studies. The experiments were carried out in rats and mice anaesthetized with ether and in guinea-pigs anaesthetized with sodium pentobarbitone, using the operative technique of Matthews, Craft, Geddes, Wise & Hyde (1968). The abdomen was opened and a length of the small intestine was isolated between ligatures. The ligated section was flushed out carefully with saline solution (2 ml) and within this length three separate sections (5 cm long) were isolated in the rat and guinea-pig. YN emulsion (0·2 ml; 5 mg/ml) was introduced into each loop through a fine needle (no. 17) and the entire length of intestine was replaced inside the abdomen. In the experiments with the mouse, the intestine of each animal was divided up into two loops and only 0·1 ml of YN emulsion was introduced into each loop.

After absorption periods of 0, 15 and 30 min, the complete length of intestine was removed from the abdomen and washed with saline, and the loops were excised and exhaustively extracted with chloroform–methanol (2:1, v/v). Aliquots of the chloroform–methanol extract were assayed for radioactivity.

In vitro *studies.* Homogenates were prepared from the washed livers of mice, guinea-pigs and ferrets killed by cervical dislocation. A 10% homogenate in Krebs–Ringer solution containing glucose (to a final concentration of 10 mM) was used for the experiments.

Homogenates of small-intestinal mucosa were prepared from the washed intestine of untreated mice, guinea-pigs and ferrets. The mucosa was scraped off the intestinal wall and homogenized in 10 vols 0·2 M-Tris-maleate buffer (pH 7·5) containing 0·5 mg desoxycholate/ml.

For the experiments, 8 ml tissue homogenate and 2 ml YN emulsion (5 mg/ml) were incubated at 37°C. Aliquots (1·0 ml) of the incubation mixture were taken at 0, 2, 6 and 24 hr, shaken with chloroform–methanol (2:1, v/v) and centrifuged. The radioactivity in both phases was measured; that in the organic phase was shown—by thin layer chromatography in chloroform–methanol–formic acid–water (70:30:7:3, v/v)—to be unhydrolysed [^{32}P]YN. At the same time, control incubations were performed under similar conditions but without tissue, and the organic and aqueous layers were analysed similarly.

Radioactivity was measured in a Nuclear-Chicago MK1 Liquid Scintillation Counter and efficiency was determined by the Channels ratio method. Quench curves for the various counting media were prepared, using the high activity [^{32}P]phosphate standard (Haviland & Bieber, 1970). All counts were corrected for natural radioactive decay.

RESULTS

Whole-animal studies

The excretion of radioactivity at various times after administration of [^{32}P]YN to male guinea-pigs and ferrets is shown in Table 1. Most of the label was excreted in the faeces within 48 hr, the ferrets clearing 60–70% in this time and the guinea-pigs between 40 and 65%. Urinary excretion of radioactivity followed a similar time course in both species. Between 5 and 10% of the dose of [^{32}P] was excreted in the first 24 hr, and thereafter the daily excretion fell to approximately 1% at day 3 and less than 0·5% by day 10. A total of between 9 and 18% of the radioactivity was eliminated by this route. Only about 1% of the radioactivity in the guinea-pig faeces was found to be extractable into chloroform–methanol.

Table 1. *Time course for the excretion of radioactivity in urine and faeces of male guinea-pigs and ferrets following a single oral dose of* [^{32}*P*]*YN*

	Radioactivity in excreta (% dose)			
	Guinea-pig		Ferret	
Day	Urine	Faeces	Urine	Faeces
1	5·25 (4·26–6·23)	28·30 (7·07–49·50)	7·54 (5·64–9·75)	36·83 (5·67–68·00)
2	2·64 (1·65–3·63)	23·43 (14·95–31·90)	1·99 (1·93–2·05)	28·16 (2·53–53·80)
3	2·05 (0·66–3·44)	5·10 (1·52–8·68)	1·27 (1·21–1·34)	2·35 (0·18–4·53)
4	0·98 (0·68–1·28)	1·12 (0·36–1·88)	ND	0·06 (0·06–0·06)
5	0·57 (0·44–0·86)		ND	0·07 (0·04–0·10)
6	0·45 (0·30–0·68)		ND	1·36 (0·18–2·55)
7 + 8	0·78 (0·47–1·09)		ND	ND
9 + 10	0·72 (0·58–0·85)	0·14 (0·13–0·14)*	ND	ND
Total....	13·44 (9·04–18·06)	58·10 (49·66–66·49)	10·81 (8·78–12·84)	(68·83 (66·65–71·05)

ND = None detected

*Total for days 5–10 inclusive.
Values are means, with ranges in parentheses.

The excretion of radioactivity after an oral dose of inorganic [^{32}P]phosphate is shown in Table 2. Faecal excretion reached approximately 60% of the given dose by 48 hr and then persisted at a relatively constant low level up to day 10. Urinary excretion accounted for approximately 10% of the dose by 48 hr and about 15% by 10 days. A considerable proportion of the dose was incorporated into the skeleton, between 15 and 20% of the dose remaining after 10 days. Comparison of the excretion data in Tables 1 and 2 shows that the time courses for both urinary and faecal excretion of [^{32}P] are similar after inorganic [^{32}P]phosphate and [^{32}P]YN.

Autoradiography

Table 3 shows the distribution of radioactivity in the mouse as determined by degrees of blackening of the autoradiographs. Although a proportion of the radioactivity passed through the gastro-intestinal tract and was excreted in the faeces, a considerable quantity of radioactivity was absorbed rapidly after dosing, as indicated by its presence in skeletal tissue and liver after only 2 hr. After 24 hr, the label was distributed over all the body tis-

Table 2. *The time-course of excretion of radioactivity in urine and faeces of male guinea-pigs following a single oral dose of* [^{32}*P*]*phosphate*

	Radioactivity (% dose) in	
Day	Faeces	Urine
1	40·21 (23·42–50·30)	9·93 (8·92–10·57)
2	12·73 (9·77–17·88)	1·83 (1·27–2·43)
3	3·63 (2·29–4·96)	1·19 (0·84–1·53)
4	0·89 (0·72–1·07)	0·81 (0·55–1·05)
5 + 6	0·63 (0·42–0·86)	1·27 (1·09–1·58)
7 + 8	0·46 (0·43–0·50)	0·69 (0·58–0·83)
9	0·23 (0·14–0·31)	
Total....	58·78 (48·93–66·09)	15·72 (14·46–17·72)

Values are means, with ranges in parentheses.

Table 3. *The distribution of radioactivity in autoradiographs of mice given a single, oral dose of* [^{32}P]*YN*

Area	Time after dosing (hr)....	Degree of blackening of film*						
		1	2	8	24	72	120	168
Skin		—	—	—	2	—	—	—
Tongue		—	—	2	2	—	—	—
Lung		—	2	—	1	—	—	—
Heart muscle		—	5	1	2	1	tr	tr
Thymus		—	4	tr	2	1	1	tr
Salivary gland		—	—	tr	1	1	tr	tr
Stomach: wall		—	—	—	1	tr	tr	tr
contents		5	—	4	4	—	1	—
Small intestine: wall		1	—	2	1	1	1	tr
contents		3	—	3	2	—	—	—
Caecum: wall		—	tr	—	1	tr	tr	—
contents		5	tr	1	3	—	—	—
Colon: wall		—	—	1	1	1	tr	tr
contents		2	1	2	3	—	—	—
Faeces		—	—	5	3	1	—	—
Liver		tr	1	1	2	1	1	tr
Kidney		—	1	1	2	1	1	tr
Urinary bladder		—	3	1	2	1	1	—
Urine		—	2	2	1	—	—	—
Testes		—	—	1	1	tr	tr	tr
Skeletal muscle		—	tr	1	2	tr	tr	tr
Spleen		—	—	1	2	1	1	tr
Spinal cord		—	—	—	1	—	tr	—
Skeleton								
Vertebral column		tr	3	5	5	5	5	5
Limb bones		tr	2	4	5	5	5	4
Pelvis		tr	2	3	5	4	4	4
Skull/jaw		tr	2	3	5	4	4	4

*Degrees of blackening: —, none after 48-hr exposure; tr, none after 24-hr, faint after 48-hr exposure; 1, faint at 24 hr; 2, obvious at 48 hr; 3, obvious at 24 hr; 4, marked at 48 hr; 5, marked at 24 hr.

sue, with particular concentrations in the bones and faeces. The radioactivity fell in the tissues during the 7 days following dosing but remained high in the skeleton. It was noticed that the activity in the bones was concentrated at the periphery and was almost absent from the centre.

Intestinal absorption

The clearance of the ^{32}P-labelled components of YN from *in situ* intestinal loops is shown in Fig. 1. Absorption from the intestine was similar in all three species studied, between 55 and 65% being cleared in 30 min. The absorption curves are clearly biphasic, with an initial steep portion, probably representing absorption into the intestinal epithelium, and a slower phase representing clearance into the circulation. *In vitro* experiments with washed segments of mouse intestine clearly demonstrated this initial phase of rapid absorption.

In vitro *hydrolysis*

Figures 2 and 3 demonstrate the [^{32}P]YN-degrading ability of the enzyme systems of the liver and intestinal mucosa of the three species investigated. In these experiments, an aliquot of the tissue homogenate was extracted with chloroform–methanol (Folch, Lees

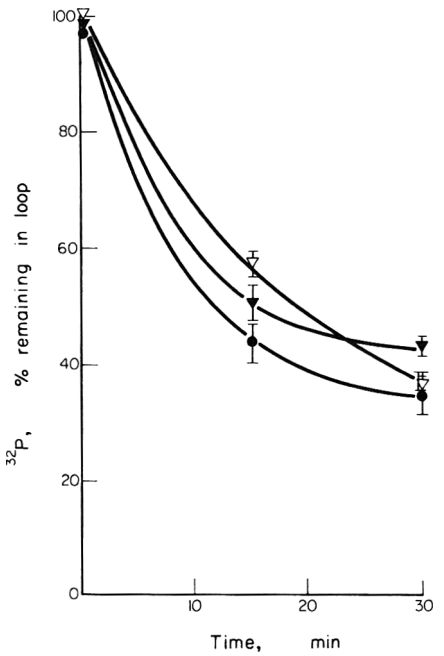

Fig. 1. Absorption of [^{32}P]YN from ligated sections of the small intestine of the rat (▽), mouse (●) and guinea pig (▼). Points are means ± SD of three loops.

& Sloane Stanley, 1957) to separate lipid and non-lipid phosphorus, the radioactivity in the organic phase being regarded as unhydrolysed [^{32}P]YN. The results may therefore somewhat underestimate the extent of hydrolysis of YN.

Incubation of [^{32}P]YN with the homogenate of intestinal mucosa of the guinea-pig resulted in a 60% breakdown of the phosphorus-containing components in 6 hr and nearly 80% breakdown in 24 hr. This was considerably greater than the hydrolysis by either mouse or ferret intestinal mucosa, the extent of breakdown being similar in both cases

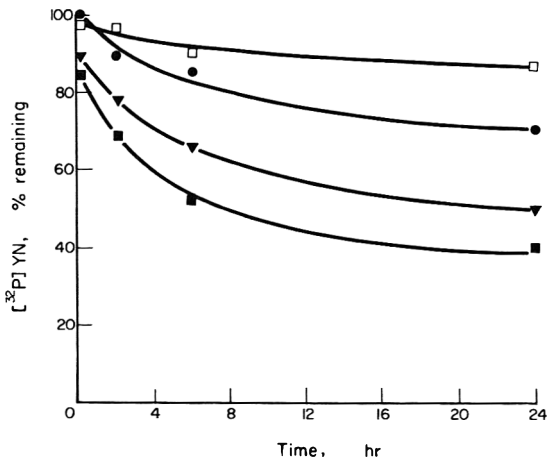

Fig. 2. Hydrolysis of [^{32}P]YN by liver homogenates of the mouse (●), guinea-pig (▼) and ferret (■), compared with that in a tissue-free control at pH 7·4 (□).

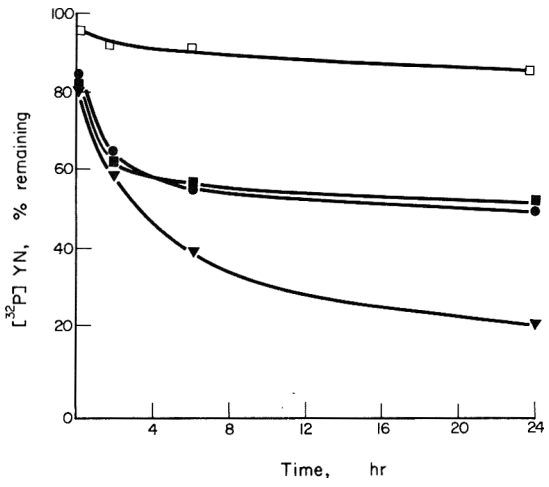

Fig. 3. Hydrolysis of [^{32}P]YN by homogenates of intestinal mucosa of the mouse (●), guinea-pig (▼) and ferret (■), compared with that in a tissue-free control at pH 7·4 (□).

(45% at 6 hr and 52% at 24 hr). The ability of liver homogenate to hydrolyse [^{32}P]YN compared with that of the intestine was not the same in each species. In both the guinea-pig and the mouse, the liver was less efficient than the intestinal mucosa (35 and 15% hydrolysis respectively at 6 hr by the liver compared with 60 and 45% by the intestine) whereas the reverse was the case in the ferret (50% by the liver in 6 hr compared with only 44% by the intestinal mucosa).

DISCUSSION

The experiments described in this report have extended the information on the metabolic fate of [^{32}P]YN to two other rodent and one non-rodent species. The excretion of radioactive phosphorus by the ferret and guinea-pig following a single oral dose of [^{32}P]YN is similar to that reported for the rat (Feuer, 1967) in that the majority of the radioactivity is cleared within 72 hr. In both the former species however, the fraction of the dose of ^{32}P appearing in the urine is significantly higher than that in the rat. This may be a reflection of the higher initial rate of hydrolysis of YN by the intestinal mucosa of the guinea-pig and ferret compared with that reported in the rat. The excretion of ^{32}P from the mouse is similarly rapid, little activity remaining in the body at 72 hr except in the skeleton. From a comparison of the time course of excretion of radioactivity and its distribution in urine and faeces after an oral dose of [^{32}P]YN or [^{32}P]phosphate to the guinea-pig, it seems likely that the majority of the dose of YN is hydrolysed in the intestinal tract.

The intestinal mucosal-cell preparations of all the species examined rapidly hydrolysed YN to inorganic phosphate, so that incorporation of YN phosphorus into the phosphate pool in the body was to be expected within a short time. This was confirmed autoradiographically in the mouse with the demonstration of radioactivity in the skeleton at 1 hr. At this time no radioactivity could be seen in any other part of the animal except the intestinal tract, apart from a trace in the liver. This is further evidence in support of the view that the major site for the metabolism of YN is the intestinal tract and that very little unhydrolysed YN is absorbed after oral administration. The *in situ* intestinal absorption studies

confirm that transfer of radioactivity into the animal is slow after the initial phase of rapid uptake by the intestinal mucosa. It seems likely, therefore, that the metabolism of YN is comparable in the rodent and non-rodent species investigated. The majority of the material is hydrolysed in the intestinal tract to inorganic phosphate and lipids, which are disposed of along normal physiological routes. Any material that is absorbed into the body is probably metabolized by the liver in a manner similar to that occurring in the intestine. Long-term storage of phosphorus-containing components of YN was not indicated by this study.

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LONG-TERM TOXICITY OF SORBIC ACID IN THE RAT

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Abstract—Groups of 48 male and 48 female rats were given diets containing 0 (control), 1·5 or 10% sorbic acid for 2 yr. There was no increase in the rate or mortality in the treated rats and no changes attributable to treatment in the haematological examinations, analyses of serum, studies of renal function or histopathological examination. The relative liver and kidney weights were increased in the rats given 10% sorbic acid. The thyroid weight was increased at the same level in males but this was not thought to be due to sorbic acid treatment. This study showed no carcinogenic effect of sorbic acid at dietary levels up to 10%. The no-effect level established by this study was 1·5% of the diet, with changes of doubtful significance at the higher level (10%).

INTRODUCTION

Sorbic acid (*trans, trans*-2,4-hexadienoic acid; $CH_3 \cdot CH{:}CH \cdot CH{:}CH \cdot COOH$) is used as a preservative in food. As indicated by the Joint FAO/WHO Expert Committee on Food Additives (1970), it is particularly useful in inhibiting the growth of fungi and yeasts and has some action against bacteria. This report also points out that the antimicrobial activity depends on the presence of the undissociated acid and as such is pH-dependent, exhibiting greatest activity under acid conditions. However, unlike many pH-dependent preservatives, sorbic acid is also active at pH values of 5–6.

Sorbic acid has preservative applications in a wide range of foodstuffs and may be incorporated into the product, sprayed on the surface or included in wrappers to give surface protection. It is particularly useful in products such as cheese and yoghurt, in which it can be used to prevent the growth of spoilage organisms without inhibiting those contributing to the natural maturing or fermentation of the product (Joint FAO/WHO Expert Committee on Food Additives, 1970).

Lishmund (1969) calculated that if sorbic acid were used at the technically acceptable levels in cheese, preserves, dried fruits and nuts, bread, cakes and pastries, pickles and sauces, wine, soft drinks and fruit or fruit pulp for manufacturing, the average daily intake would be 293 mg. The major part of this, 222 mg/day, would be contributed as a result of use in bread. Even at this level of use the intake would be below the unconditional acceptable daily intake of 12·5 mg/kg (750 mg/day for a 60-kg adult) set by the Joint FAO/WHO Expert Committee on Food Additives (1967).

The use of sorbic acid in foodstuffs is permitted to varying degrees in a wide range of countries, including the USA (Sec. 121.101 of the Code of Federal Regulations). In the UK, the use of sorbic acid has been limited to levels of up to 1000 ppm in cheese, flour confectionery, solutions of food colourings and silicone antifoam emulsions (The Preservatives in Food Regulations, 1962, Statutory Instrument 1962, no. 1532). This use was reviewed in the report of the Food Additives and Contaminants Committee (1972) and it was recommended that the permitted use of this perservative should not be extended, as the data

from long-term studies were inadequate. Sorbic acid has a low order of acute toxicity, the LD_{50} values after oral administration in rats being in the range 7·36–10·5 g/kg for the free acid and 4·0–7·16 g/kg for the potassium salt (Smyth & Carpenter, 1948; Deuel, Alfin-Slater, Weil & Smyth, 1954a). In mice the LD_{50} values were 2·8 g/kg after ip injection and in excess of 8 g/kg after oral administration (Sparfel, Lafille & Le Reste, 1968). In short-term studies in rats (90–120 days duration), the only effects reported have been reduced rates of body-weight gain and increases of liver weight, without any histopathological abnormality, with dietary levels of 8% (Deuel *et al.* 1954a) or 10% (Demaree, Sjogren, McCashland & Cosgrove, 1955). In the latter study treatment had no influence on the number of young animals born in a reproduction study. A dietary level of 4% given over 3 months produced no adverse effects in dogs (Deuel *et al.* 1954*a*).

In long-term studies in rats, no histopathological changes nor increases in tumours were produced by dietary levels of up to 5% sorbic acid (W. Kieckebusch, K. Lang and W. Griem, unpublished data 1967, provided by Hoechst Chemicals Ltd.), daily intakes of 40 mg/kg/day (Shtenberg & Ignat'ev, 1970) or 0·1% in the drinking-water (Dickens, Jones & Waynforth, 1966). The only effects were an increased rate of body-weight gain, attributed to a higher calorific content of the diet, and increases of liver and kidney weights in rats given 5% sorbic acid in the diet (W. Kieckebusch, K. Lang and W. Griem, unpublished data 1967). Daily intakes of up to 80 mg sorbic acid/kg/day were without effect (Shtenberg & Ignat'ev, 1970). These authors concluded that sorbic acid was the least toxic of a number of preservatives studied and their recommendation that it should replace benzoate and sulphite wherever possible is being followed in the USSR.

On repeated sc injection, sorbic acid, dissolved in either oil or water, led to subcutaneous sarcoma formation in rats, although there were differences with various batches of sorbic acid. However, the potassium salt was without effect in this respect (Dickens *et al.* 1966; Dickens, Jones & Waynforth, 1968; Dickens & Waynforth, 1967). No sarcomas were produced when mice were injected with sorbic acid (Gericke, 1968). A study of the type of reactions produced in the subcutaneous tissue of the rat following a short series of injections led Grasso, Gangolli & Hooson (1969) to conclude that sorbic acid, either in aqueous solution or in oil, was likely to lead to sarcoma production. They suggested that the reaction seen resulted from local changes of pH, repeatedly causing necrosis followed by regeneration. This cycle of events was typical of materials causing local sarcoma due to the physico-chemical properties of the injected solutions and the authors considered that the sarcomas seen after sorbic acid injection were not an indication of a carcinogenic effect.

The metabolism of sorbic acid is similar to that of analogous saturated fatty acids as has been shown using *in vitro* systems (Cohen, 1937; Deuel, Calbert, Anisfeld, McKeehan & Blunden, 1954b; Lang, 1960; Mahler, Wakil & Bock, 1953; Witter, Newcomb & Stotz, 1950). In the intact animal, sorbic acid, at dietary levels up to 5%, did not act as an anti-metabolite for essential fatty acids or accelerate the depletion of these fatty acids in animals maintained on a fat-free diet (Deuel *et al.* 1954a). The degree of urinary ketone excretion and its suppression by carbohydrate was similar in rats treated with sorbic acid or its saturated analogue, caproic acid (Deuel *et al.* 1954b).

Studies with labelled sorbic acid in rats (Fingerhut, Schmidt & Lang, 1962) and mice (Westöö, 1964) showed that most of an oral dose was rapidly absorbed and metabolized, with over 80% of the radioactivity being present as carbon dioxide in the expired air. The half-life for sorbic acid was 40–110 min in the rat and 2–8 hr in the mouse. Approximately

2–4% of the activity of the dose was present in the urine, in the form of carbon dioxide and urea in the rat but as sorbic acid and *trans*, *trans*-muconic acid in mice. Glycogen was unlabelled but there was some activity in the body fat, suggesting that breakdown products of sorbic acid are re-utilized in lipid synthesis. In a parallel study with caproic acid, Fingerhut *et al.* (1962) demonstrated a similar fate for the saturated acid.

In view of the lack of adequate data from long-term observations in animals, particularly with reference to the possible carcinogenic effects of sorbic acid, such studies have been undertaken as part of the safety evaluation programme at BIBRA. This paper presents the results of a long-term study in the rat.

EXPERIMENTAL

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

Animals and diet. Rats of the Wistar strain obtained from an SPF breeding colony were housed four to a cage in an animal room maintained at 20 ± 1°C with a relative humidity of 50–60%. The basic diet, Spillers' Laboratory Small Animal Diet, and tap-water were provided *ad lib.*

Experimental design and conduct

Groups of 48 male rats (body weight 90–145 g) and 48 females (body weight 80–130 g) were fed diets containing 0 (control), 1·5 or 10% sorbic acid. In order to provide similar calorific intakes in all groups, a mixture of 50% corn oil and 50% starch (by weight) was added to the control and 1·5% sorbic acid diets. This mixture was added at a rate of 10% to the control diet and 8·5% to the diet containing 1·5% sorbic acid.

Body weight, food consumption and water consumption were recorded initially, after 1 month and thence at 3-monthly intervals. The rats were observed for general condition and health and any rat that appeared to be ill was isolated, to be returned to its cage if its condition improved or to be killed if its condition deteriorated. All animals dying or killed during the course of the study were subjected to a post-mortem examination unless this was precluded by autolysis or cannibalism. Samples of the tissues were preserved for histopathological examination.

At the end of the study all surviving rats were killed by an overdose of barbiturate following an overnight fast. At the post-mortem examination all macroscopic abnormalities were noted and the brain, pituitary, heart, liver spleen, kidneys, stomach, small intestine, caecum, gonads, adrenal glands and thyroid were weighed. Samples of these tissues and of salivary gland, thymus, various lymph nodes, pancreas, urinary bladder, aorta, lung, trachea, colon, rectum, skeletal muscle, spinal cord and uterus, together with any other tissue

that appeared to be abnormal, were preserved in 10% buffered formalin. Paraffin-wax sections of these tissues were stained with haematoxylin and eosin for histopathological examination.

Blood samples were collected from the tail vein of ten male and ten female rats from each group at wk 12, 27, 52 and 80. Samples were also collected, from the aorta of all surviving rats, during autopsy at 2 yr. The samples collected up to wk 52 were examined for haemoglobin concentration, packed cell volume, total erythrocyte count and total leucocyte count. At wk 80 and 2 yr these examinations were confined to measurement of the haemoglobin concentration. Slides for differential white counts and reticulocyte counts were prepared from all blood samples but only those from the controls and the animals given diet containing 10% sorbic acid were examined.

Serum collected from the surviving rats at 2 yr was analysed for the content of glucose, urea, total protein, albumin and the activities of glutamic–oxalacetic transaminase, glutamic–pyruvic transaminase and lactic dehydrogenase. Urine samples, collected from ten rats of each sex at wk 13, 26 and 52, were analysed for content of glucose, bile, blood, protein and ketones. A count of the number of cells excreted was carried out and a concentration test was performed. The latter consisted of making measurements of the volume and specific gravity of the urine produced in a 6-hr period of water deprivation, in a 2-hr period immediately following a water load of 25 ml/kg and in a 4-hr period commencing 16 hr after the water load.

RESULTS

Apart from the changes of appearance regularly occurring in ageing rats, no abnormalities were seen in the rats given sorbic acid in their diet. The numbers of animals killed or found dead were similar in each group and in the females the highest number of deaths was in the control group. Most of the rats died or were killed during the last 6 months of the study, the overall mortality at wk 80 being 8% in males and 3% in females, while the corresponding figures at wk 92 were 20 and 8% and at wk 102, 40 and 24%. The majority of the animals were therefore exposed to the treatment for at least 21 months. Most of the deaths were in animals killed because of ill health, only 19 and 6% of the total males and females, respectively, being found dead in their cages.

The rate of body-weight gain of the rats given 1·5% sorbic acid did not differ significantly from that of the controls (Table 1). In the rats given 10% sorbic acid, the rate of body-weight gain was less than in the controls and the differences were statistically significant from wk 26 onwards in the females and from wk 39 onwards in the males. Despite this statistical significance, the difference of body weight was relatively small, being 5–10% of the control weight.

There were no consistent differences between the treated and control rats in food consumption (Table 1), although there were some statistically significant decreases, particularly at the top level, during the second half of the study. Water consumption was comparable in all groups. Calculation of the levels of intake of sorbic acid over the experimental period established a mean daily intake of 0·63 and 4·33 g/kg in males and 0·85 and 5·69 g/kg in females given dietary levels of 1·5 and 10% respectively.

The findings in the haematological examinations are shown in Table 2 for wk 52 and 104. No adverse effects were evident in the treated groups at these times or at wk 12 and 80. There was a statistically significant reduction ($P < 0·01$) in the total leucocyte count in the females given 10% sorbic acid for 27 wk, but no similar change was found in the males

Table 1. *Body weight and food consumption of rats fed diets containing 0, 1·5 or 10% sorbic acid for 2 yr*

Sex and dietary level (%)	Value at wk									
	0†	4	13	26	39	52	65	78	92	101
Male	**Body weight (g)**									
0	127	313	459	550	627	693	748	779	755	730
1·5	125	313	467	558	630	709	749	775	758	727
10·0	127	299	442	532	585**	655**	684**	698***	673**	675
Female										
0	114	207	268	302	335	379	418	444	462	494
1·5	113	203	259	294	324	372	414	445	475	459
10·0	113	204	258	286*	313**	351**	383**	407*	421*	429***
Male	**Food consumption‡ (g/rat/day)**									
0	16·4	19·8	18·2	19·3	24·2	19·0	20·0	21·7	19·0	17·5
1·5	17·1	19·8	18·9	20·2	24·8	19·9	17·6**	18·7**	19·4	19·3
10·0	16·6	20·0	18·8	19·0	22·5*	19·9	16·8**	20·1	17·6	19·5
Female										
0	15·0	14·6	13·5	14·5	21·8	16·6	16·6	19·1	18·8	18·3
1·5	14·4	15·4	13·9	13·9	20·8	17·6	16·3	17·5	20·5	17·9
10·0	14·0	16·2	13·6	14·2	20·3	17·4	13·3*	17·2*	16·7	17·9

† Value on first day of study (prior to treatment).
‡Food consumption was measured over a period of 48 hr.
The figures for body weight are the means for all surviving rats in each group and those for food consumption are the means for 12 cages each initially containing four rats. Values marked with asterisks differ significantly (Student's *t* test) from those of controls: $*P < 0{\cdot}05$; $**P < 0{\cdot}01$; $***P < 0{\cdot}001$.

at the same time or in either sex at the later examination. There was no effect on the proportion of the different types of leucocyte present.

The only abnormality in the serum analyses was an elevation ($P < 0{\cdot}05$) in the concentration of urea in the males given 10% sorbic acid. However, examination of the data showed that there were two high values (75 and 90 mg/100 ml) and without these the mean was reduced from 31 to 25 mg/100 ml, a figure not significantly different from the controls.

No abnormal constituents were present in the urine at the three examinations carried out during yr 1 of the study, and the number of cells excreted in the urine (Table 3) was similar in treated and control animals. In the concentration test, the only difference from control values was a slightly increased urinary volume over a 6-hr collection period in the case of the females given 10% sorbic acid for 13 or 52 wk. There was no parallel finding at wk 26 in the females or in males at any time.

The only difference between treated and control rats in the organ weights was a higher thyroid weight ($P < 0{\cdot}01$) in the male rats given 10% sorbic acid. This difference was also evident when the organ weights were expressed relative to body weight (Table 4). In addition, there were higher values for the relative liver weight of both sexes given 10% sorbic acid and for the relative kidney, small intestine and ovary weights in the females at the same dose.

There was a wide variety of histopathological findings in all of the groups (Table 5). In the male rats the incidence of these was not adversely influenced by treatment with sorbic acid. In the females the incidences of some of the lesions were altered in the rats given 10% sorbic acid. There were increased incidences of haemosiderosis of the spleen and focal

Table 2. *Haematological findings in rats fed diets containing 0, 1·5 or 10% sorbic acid for 52 or 104 wk*

Sex and dietary level (%)	No. of rats examined	Hb (g/100 ml)	PCV (%)	RBC (10^6/mm^3)	Retics (% of RBC)	Leucocytes Total (10^3/mm^3)	Leucocytes Differential (%) N	E	L	M
Male				**Wk 52**						
0	10	15·7	52	7·16	1·02	11·00	17	1	80	2
1·5	10	15·6	52	7·08	—	10·18	—	—	—	—
10·0	10	14·5	50	7·15	1·15	9·27	16	2	80	2
Female										
0	10	15·7	46	6·03	1·13	7·86	13	2	82	3
1·5	10	15·5	47	6·97**	—	7·00	—	—	—	—
10·0	10	15·2	45	5·92	0·99	7·25	17	2	79	2
Male				**Wk 104**						
0	29	14·4	—	—	—	—	48	2	49	1
1·5	32	13·8	—	—	—	—	—	—	—	—
10·0	21	13·5	—	—	—	—	39	2	56	3
Female										
0	31	14·1	—	—	—	—	43	2	53	2
1·5	39	13·9	—	—	—	—	—	—	—	—
10·0	36	13·7	—	—	—	—	41	2	55	2

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

The figures are means for the numbers of rats shown and that marked with asterisks differs significantly ($P < 0·01$ by Student's t test) from the corresponding control figure.

Table 3. *Results of renal concentration tests and renal cell excretion of rats given diets containing 0 or 10% sorbic acid for up to 1 yr*

Sex and dietary level (%)	Cell excretion (10^3/hr)	Concentration test Specific gravity 0–6 hr	Specific gravity 16–20 hr	Volume (ml) 0–6 hr	Volume (ml) 16–20 hr	Dilution test (2 hr) Specific gravity	Volume (ml)
Male			**Wk 13**				
0	5·6	1·050	1·072	2·2	0·9	1·009	7·0
10	2·8	1·055	1·072	1·7	0·7	1·008	7·2
Female							
0	2·6	1·052	1·070	0·5	0·6	1·014	2·6
10	2·9	1·034	1·072	1·5**	0·6	1·009	3·9
Male			**Wk 52**				
0	6·8	1·052	1·081	2·4	0·3	1·004	9·1
10	4·9	1·062	1·084	2·6	0·3	1·005	7·3
Female							
0	3·5	1·051	1·077	0·9	0·4	1·002	10·4
10	2·2	1·042	1·071	1·9**	0·2	1·002	9·0

The figures are the means for groups of ten rats. Those marked with asterisks differ significantly (ranking method of White, 1952) from those of controls: $**P < 0·01$. No significant differences were found in the test animals at wk 26.

Table 4. *Organ weights and relative organ weights of rats fed diets containing 0, 1·5 or 10% sorbic acid for 2 yr*

Sex and dietary level (%)	No. of rats examined	Body weight (g)	Relative weights (g/100 g body weight) of											
			Brain	Heart	Liver	Spleen	Kidneys	Stomach	Small intestine	Caecum	Adrenals†	Gonads‡	Pituitary†	Thyroid†
Male														
0	29	702	0·32	0·24	2·40	0·26	0·63	0·46	1·47	0·24	12·3	0·47	2·0	5·8
1·5	32	702	0·31	0·24	2·50	0·25	0·65	0·44	1·48	0·24	12·2	0·47	2·0	5·7
10·0	26	656	0·34	0·25	2·64**	0·25	0·82	0·50	1·62	0·25	12·4	0·52	2·0	7·7*
Female														
0	36	452	0·45	0·28	2·66	0·24	0·63	0·54	1·87	0·35	18·0	24·1	4·1	7·9
1·5	39	431	0·45	0·28	2·70	0·24	0·61	0·56	2·01	0·33	16·8	27·5	4·2	8·3
10·0	38	410	0·48	0·30	2·99**	0·26	0·72*	0·57	2·08*	0·33	17·9	29·3*	4·0	7·5

†Values for these organs are expressed in mg/100 body weight.
‡Values for female gonads are expressed in mg/100 g body weight.
Figures are the means for the numbers of rats shown and those marked with asterisks differ significantly (Student's t test) from those of controls: $*P < 0·05$; $**P < 0·01$.

Table 5. *Findings in the histopathological examination of rats fed diets containing 0, 1·5 or 10% sorbic acid for 2 yr*

Tissue and lesion	Incidence of lesions in: Males given dietary levels (%) of 0	1·5	10·0	Females given dietary levels (%) of 0	1·5	10·0
No. of rats examined . . .	43	43	41	42	45	46
Thyroid						
Large vesicles containing colloid	3	6	2	0	1	0
Numerous small follicles	3	4	7	4	2	5
Hyperplasia of parathyroid	9	10	4	1	4	1
Adrenal glands						
Hyperplasia	1	0	0	0	0	2
Cortical cell vacuolation	13	15	12	6	5	9
Cysts	0	0	0	7	7	4
Pituitary						
Hyperplasia	4	0	1	8	10	7
Haemorrhage	0	0	0	18	17	18
Pancreas						
Hyperplastic nodule	3	0	0	0	0	0
Giant islet	2	2	1	0	1	0
Heart						
Myocardial fibrosis	24	25	23	13	9	15
Foci of calcification	4	1	7	1	0	0
Medial hypertrophy of coronary arteries	2	0	1	4	1	2
Arteries						
Periarteritis	6	4	1	2	5	4
Calcification	3	1	1	0	0	0
Kidney						
Glomerulonephrosis	31	24	28	33	39	37
Marked glomerulonephrosis	10	17	11	10	3	9
Mineral deposits	5	0	1	1	0	1
Hydronephrosis	0	0	1	0	0	0
Leucocyte infiltration	0	0	2	2	0	0
Liver						
Focal fatty change	16	19	21	13	14	32
Marked fatty change	9	6	5	12	5	8
Bile-duct hyperplasia	11	16	7	29	24	5***
Focal necrosis	5	5	2	0	2	10**
Nodular hyperplasia	0	0	0	0	1	2
Portal fibrosis	3	0	2	3	5	1
Extramedullary haematopoiesis	1	0	0	0	0	2
Haemorrhage	0	0	0	3	0	4
Haemangioma	0	1	0	3	2	7
Spleen						
Increased extramedullary haematopoiesis	20	10	3*	10	18	16
Haemosiderin deposition	3	1	0	6	9	18
Lymph nodes						
Haemorrhage	3	4	3	4	3	7
Cystic	0	1	1	1	0	1
Hyperplasia	0	0	1	2	0	0
Thymus						
Haemorrhage	1	0	1	0	0	0
Cystic	1	2	1	0	1	0
Hyperplasia	1	0	0	4	2	1

Table 5. *Continued*

Tissue and lesion	No. of rats examined . . .	Incidence of lesions in Males given dietary levels (%) of 0	1·5	10·0	Females given dietary levels (%) 0	1·5	10·0
		43	43	41	42	45	46
Lung							
Alveoli containing foamy macrophages		9	8	6	7	10	7
Leucocyte infiltration		2	7	8	2	4	7
Congestion		2	1	2	0	0	2
Emphysematous areas		1	2	2	1	0	0
Granuloma		5	7	2	2	4	6
Stomach							
Hyperkeratosis		0	0	1	1	0	0
Dilated gastric glands		3	4	6	2	2	4
Calcium deposits in mucosa		2	3	3	0	0	0
Hyperplasia of mucosa		0	1	1	3	0	0
Ileum							
Mucocele		0	0	0	0	1	0
Increased lymphoid tissue		3	3	0	0	0	0
Necrotic area		0	1	0	0	0	0
Duodenum							
Mucus cysts		0	0	1	0	0	0
Skeletal muscle							
Calcium deposition		2	0	2	0	0	0
Fibrosis		0	0	0	0	1	1
Skin							
Ulcer		2	1	0	0	2	0
Salivary glands							
Dilated ducts		0	0	1	1	1	0
Testes							
Atrophy		17	10	11	—	—	—
Periarteritis		6	8	8	—	—	—
Ovaries							
Cysts		—	—	—	7	5	8
Haemorrhage		—	—	—	1	1	2
Uterus							
Hydrometra		—	—	—	0	1	1
Granulation tissue		—	—	—	1	1	1
Endometrial hyperplasia		—	—	—	4	7	11

The figures represent the number affected out of the total number of rats shown. The incidences marked with asterisks differ significantly (chi-squared test) from those of the controls: $*P < 0{\cdot}05$; $**P < 0{\cdot}01$; $***P < 0{\cdot}001$.

fatty change of the liver. In both of these the degree of the change was very minor, being barely discernible from an appearance that would be considered normal. In this same group the incidence of focal necrosis of the liver was increased to a statistically significant degree but, on the other hand, the number of rats showing bile-duct hyperplasia was reduced compared with the controls.

The tumour incidence is shown in Table 6. The total incidence, the incidence of malignant tumours and the distribution of affected tissues were not influenced by sorbic acid treatment. In fact, no malignant tumours were found in the females given 10% sorbic acid. In

many cases the incidence of the tumours was similar in the control and treated animals or the tumours occurred only in the control rats.

Some tumours were found in treated rats without any similar findings in the controls. Of these, five in the males (three islet-cell tumours of the pancreas, one phaeochromocytoma and one medullary adenoma of the adrenal) and four in the females (two mammary adenocarcinomas, one renal adenocarcinoma and one fibroma of the subcutaneous tissue) were found in the rats given 1·5% sorbic acid with no similar lesions in those given 10%. In the male rats there were one or two thyroid adenomas, squamous-cell carcinomas, reticulum-cell neoplasms and pancreatic exocrine adenomas in both groups given sorbic acid, but in none of these was the incidence increased with the higher dose. The only tumours occurring solely at the higher dose were an adenocarcinoma of the salivary gland, an adenocarcinoma of the prostate and a subcutaneous fibrosarcoma in males and a lipoma in the females.

DISCUSSION

The observation of a reduced rate of body-weight gain in the male rats given diet containing 10% sorbic acid may suggest some slight non-specific toxic effect. In the past, increased body weights have been reported in rats given diets containing 4–5% sorbic acid (Deuel *et al.* 1954a; W. Kieckebusch, K. Lang and W. Griem, unpublished data 1967) but decreased weights were found with dietary levels of 8% (Deuel *et al.* 1954a). The authors suggest that the increased rates of weight gain were due to the calorific contribution of sorbic acid, a factor that was controlled in the present study by increasing the calorific content of the basal diet. Thus the system used in these studies is probably more sensitive for detecting any adverse effects of sorbic acid on body-weight gain. Nevertheless the difference from the controls was small (5–10% of the control body weight) and was associated with some reductions in food intake. It is not possible with the available data to determine whether the slight reduction of weight gain was due to unpalatable diet. However, as the mortality was not affected, even when the material was fed over the greater part of the lifespan, it seems unlikely that this represented a serious toxic effect.

The finding of elevated blood urea in the male animals given 10% sorbic acid is related to the normal ageing changes in the rat kidney rather than to any effect of the preservative. It was found that the difference from the controls was due to particularly high values in two rats and autopsy and histopathology revealed that these two animals had particularly severe glomerulonephrosis. Various degrees of glomerulonephrosis are commonly found in the kidneys of ageing rats (Berg, 1967) and the lesion is associated, in its terminal stages, with an increased blood-urea level (Berg, 1965). Thus, the apparent elevation of urea levels found in this study was due to the presence, at the time of sampling, of two animals suffering from the terminal stages of normal degenerative renal changes.

In the analysis of organ weights the only organ showing an increase in terms of absolute weight was the thyroid of the male rats given 10% sorbic acid, no similar change being evident in the females. An examination of the individual data showed that there were nine rats with thyroid weights in excess of the control range. All of these animals had, in addition, one or more of the following changes: microscopically obvious parathyroid hyperplasia (two rats), markedly increased kidney weights (six rats), obvious macroscopic changes of the kidney associated with glomerulonephrosis (eight rats), microscopic evidence of severe glomerulonephrosis (three rats) and high serum-urea levels (two rats). On this basis all of the animals with increased thyroid weights showed some signs of advanced

Table 6. *Incidence of tumours found in rats fed diets containing 0, 1·5 or 10% sorbic acid for 2 yr*

Organ and tumour	Incidence* of tumours in Males given dietary levels (%) of			Females given dietary levels (%) of		
	0	1·5	10·0	0	1·5	10·0
No. of rats examined . . .	43	43	41	42	45	46
Salivary gland						
Adenoma	1	0	0	0	0	0
Adenocarcinoma	0	0	1	0	0	0
Brain						
Glioma	1	0	0	0	0	0
Thyroid						
Adenoma	0	1	1	0	0	0
Adenocarcinoma	0	0	0	1	0	0
Skin						
Squamous cell carcinoma	0	2	1	0	0	0
Pancrease						
Islet cell tumour	0	3	0	0	0	0
Exocrine adenoma	0	2	1	0	0	0
Prostate						
Adenocarcinoma	0	0	1	—	—	—
Thymus						
Lymphoma	0	0	1	3	1	2
Thymoma	0	0	0	1	0	0
Adenocarcinoma	0	0	0	1	0	0
Ovary						
Granulosa cell tumour	—	—	—	1	0	0
Testis						
Interstitial cell adenoma	2	2	5	—	—	—
Uterus						
Fibroid	—	—	—	1	0	0
Mammary gland						
Adenofibroma	—	—	—	7	11	3
Adenocarcinoma	—	—	—	0	2	0
Stomach						
Papilloma	0	0	0	1	0	0
Kidney						
Adenocarcinoma	0	0	0	0	1	0
Adrenal gland						
Cortical adenoma	1	0	0	1	0	1
Phaeochromocytoma	0	1	0	0	0	0
Medullary adenoma	0	1	0	0	0	0
Pituitary						
Adenoma	9	7	6	17	14	10
Harderian gland						
Adenoma	1	0	0	0	0	0
Ileum						
Mucoadenocarcinoma	1	0	0	0	0	0
Subcutaneous tissue						
Fibroma	1	1	1	0	1	0
Fibrosarcoma	0	0	1	0	0	0
Fat						
Lipoma	0	0	0	0	0	1

Table 6. *Continued*

Organ and tumour	No. of rats examined ...	Incidence* of tumours in Males given dietary levels (%) of 0	1·5	10·0	Females given dietary levels (%) of 0	1·5	10·0
		43	43	41	42	45	46
General							
Lymphosarcoma		3	0	1	1	0	0
Reticulum cell neoplasm		0	1	1	0	0	0
Total tumours		20	21	21	35	30	17
Tissues involved		9	8	10	9	5	5
Malignant tumours		5	3	6	3	3	0
Tissues involved		3	2	5	3	2	0

*No. of rats affected in total no. examined.

renal changes. Snell (1967) summarized work showing that prolonged renal damage in the rat can lead to parathyroid hyperplasia probably as an attempt to maintain the calcium-phosphorus ratio in the face of a reduced renal phosphate excretion. It is noteworthy therefore that at least two of the animals with unusually heavy thyroids also had hyperplastic parathyroids. It is concluded that the heavier thyroids do not represent an effect of sorbic acid on the thyroid but rather an indirect effect of renal damage on the parathyroid. This would be in keeping with the effect being present in males only, as glomerulonephrosis is usually less severe in females (Snell, 1967).

In rats given 10% sorbic acid there was an increased relative kidney weight, a finding also reported by W. Kieckebush, K. Lang and W. Griem (unpublished data 1967) using a dietary level of 5%. In the present study there were also some increases in the volume of urine excreted by rats given 10% sorbic acid. However, this increased volume of urine was not found consistently throughout the experiment. It is doubtful to what extent this indicates impaired renal function in ageing rats, particularly as it was not associated with any exacerbations in the histopathology of the kidney.

There were also slight increases in relative liver weight in the rats given 10% sorbic acid, as has previously been reported in rats given sorbic acid at dietary levels of 8% (Deuel *et al.* 1954a), 10% (Demaree *et al.* 1955) or 5% (W. Kieckebusch, K. Lang and W. Griem, unpublished data 1967). In the previous studies this slight increase was not associated with any histopathological change and might well have represented an increased metabolic demand due to the high level of fatty acid. In the present study there was no evidence of any histopathological change in the male animals but in the females there was an increased incidence of marginal changes such as mild fatty change and focal necrosis. The fatty change could have resulted from an increased intake of fatty acids (Ashworth, Sanders & Arnold, 1961). The focal necrosis may have been an indication of an incidental infection, probably of viral origin, found more frequently in this group of rats. It is obvious that the feeding of sorbic acid at 10% of the diet, although increasing the relative liver weight slightly, has no definite hepatotoxic effect as seen by the histopathological examination.

Many of the tumours in the present study were found in similar numbers in treated and control rats and cannot be attributed to any influence of sorbic acid. Other tumours occurred only in the animals given 1·5% sorbic acid and their absence at the higher level makes it unlikely that the test material was responsible for their formation.

In addition, the types of tumours seen in these animals have been reported in untreated rats, again suggesting that their presence was not related to sorbic acid treatment. For example, pancreatic islet-cell tumours have been seen in control rats in other studies in these laboratories (Brantom, Gaunt, Hardy, Grasso & Gangolli, 1973; Gaunt, Brantom, Grasso, Creasey & Gangolli, 1972a) as have adenomas of the adrenal (Gaunt *et al.* 1972a; Gaunt, Carpanini, Grasso & Lansdown, 1972b), whilst phaeochromocytomas have been reported by Rowlatt (1967) and by Snell (1967). Similarly the tumours found only in females given 1·5% sorbic acid are known to occur spontaneously. Using the same strain of rat in these laboratories, Brantom *et al.* (1973) found mammary adenocarcinoma and subcutaneous fibroma in untreated female rats, and renal tumours, though rare, have been described in untreated rats (Snell, 1967) and have been seen in these laboratories in rats given non-carcinogenic chemicals (Gaunt *et al.* 1972b).

Some tumours were found in both groups of rats given sorbic acid but the incidences were either the same at both dietary levels or higher in the rats given the lower dose (1·5%). This suggests that the occurrence of these lesions was random rather than associated with treatment and this is supported by the fact that the tumours were of types known to occur spontaneously in rats. For example, thyroid adenoma and pancreatic exocrine adenoma were observed in controls of a similar experiment in these laboratories (Brantom *et al.* 1973) and squamous cell carcinoma has occurred in these laboratories independent of treatment (Brantom *et al.* 1973; Gaunt *et al.* 1972b) and has been reported by Snell (1965). Reticulum-cell neoplasms have been reported by Lemon (1967) and Snell (1965).

The tumours found only at the highest level of treatment were not confined to any particular tissue and were found only in individual cases. This, and the fact that they were all tumours of known spontaneous occurrence suggests that the fact that they were found in the treated animals only was due solely to chance. Of these tumours, fibrosarcomas of the connective tissue and lipomas were found with a higher incidence in control animals in a concurrent experiment (Brantom *et al.* 1973). Spontaneous malignant tumours of the prostate in the rat appear to be uncommon but have been reported (Franks, 1967), and a similar lesion was found in a treated rat from a low-dose group in an experiment in these laboratories concurrent with the present study (I.F. Gaunt, Joan Hardy and P. Grasso, unpublished information 1973). Salivary gland tumours similarly appear to be rare but not unknown (Snell, 1965) and have been encountered randomly in these laboratories (Brantom *et al.* 1973; Gaunt *et al.* 1972b).

It is concluded that this study has failed to detect any carcinogenic effect on the part of sorbic acid when fed at levels of up to 10% of the diet. This represents an intake of approximately 5 g/kg/day so that even with the usual 100-times safety factor an acceptable daily intake on the basis of carcinogenicity would be 50 mg/kg/day, a value in excess of the unconditional level set by the Joint FAO/WHO Expert Committee on Food Additives (1967). This lack of effect is in keeping with earlier studies (W. Kieckebusch, K. Lang and W. Griem, unpublished data 1967).

There were minor changes in the livers and kidneys of the rats given 10% sorbic acid although these cannot definitely be attributed to the test material. No effects were found at a dietary level of 1·5%, establishing this as a no-effect level equivalent to an intake of approximately 750 mg/kg/day. However, the doubtful nature of the effects at the higher level indicate that the no-untoward-effect level may be near 5%. On the basis of these results the acceptable daily intake may be higher than the 12·5 mg/kg established by the Joint FAO/WHO Expert Committee on Food Additives (1967).

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EFFECT OF PARAQUAT ON THE BIOSYNTHESIS OF DEOXYRIBONUCLEIC ACID, RIBONUCLEIC ACID AND PROTEIN IN THE RAT

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Abstract—This study was designed to determine the relative subcellular distribution of paraquat and the effect of paraquat on the biosynthesis of DNA, RNA and protein in rat lung, liver and kidney. Biochemical parameters were correlated with the clinical syndrome. Paraquat was found in all the major cellular fractions at various times after administration of 126 mg/kg by gavage and no evidence of significant accumulation in any subcellular fraction was present. L-Leucine incorporation into protein was increased ($P < 0{\cdot}05$) in the lung, liver and kidney 32 hr after paraquat administration, and in the lung, protein synthesis was still increased at 128 hr ($P < 0{\cdot}05$). DNA biosynthesis was significantly decreased in the three organs 32 hr after paraquat treatment. In the kidney, this reduction was also significant at 64 hr. The rate of RNA synthesis was significantly decreased at 16 hr in the liver and kidney. The biochemical changes correlated in time with the histopathological changes induced by paraquat and preceded clinical signs of toxicity by 1–2 days.

INTRODUCTION

Ingestion of the herbicide paraquat (1,1′-dimethyl-4,4′-bipyridylium dichloride) by man and animals produces progressive respiratory failure manifested clinically as dyspnoea, hypoxaemia and hyperpnoea. Post-mortem examination usually reveals central zonal necrosis of the liver and cellular necrosis of the renal proximal tubules. However, the most prominent lesions are in the lungs (Clark, McElligott & Hurst, 1966; Kimbrough & Gaines, 1970) and include atelectasis, occasional hyaline membranes, congestion, oedema, haemorrhage and extensive interstitial fibrosis (Fowler & Brooks, 1971; Manktelow, 1967; Murray & Gibson, 1972; Robertson, Enhörning, Ivemark, Malmqvist & Modée, 1971). The progressive fibrosis and alveolar epithelial proliferation produced by paraquat resembles the Hamman-Rich syndrome and the lesions produced by oxygen toxicity or physical irritants.

Paraquat accumulated transiently in the lungs of rats following administration orally (Murray & Gibson, 1974) or iv (Sharp, Ottolenghi & Posner, 1972) and reached a peak concentration in the lungs 32 hr after administration. In contrast, serum paraquat levels declined rapidly, reaching a low plateau level 4 hr after administration. Paraquat was excreted in both urine and faeces, the faecal route being involved mainly with the removal of unabsorbed herbicide following oral administration (Murray & Gibson, 1974). Prolonged excretion and measurable tissue residues of paraquat suggested strong tissue binding of a small fraction of the administered dose (Murray & Gibson, 1974; Pasi & Hine, 1971). No paraquat metabolites were detected in the urine of rats (Conning, Fletcher & Swan, 1969; Murray & Gibson, 1974), although paraquat metabolites were probably formed as a result of microbiological degradation in the gut and were reported in faecal homogenates (Daniel & Gage, 1966). Instillation of paraquat into lung and skeletal muscle

caused local fibrosis suggesting that paraquat, rather than a metabolite, was responsible for the toxic changes (Kimbrough & Gaines, 1970).

A decrease in pulmonary surfactant has been suggested as the mechanism for the pulmonary toxicity of paraquat (Malmqvist, Grossmann, Ivemark & Robertson, 1973; Manktelow, 1967). However, the lungs of paraquat-poisoned animals produced no evidence of any effect on major phospholipids (Fletcher & Wyatt, 1970) or of any change in the amount or rate of destruction of dipalmitoyl lecithin (Fletcher & Wyatt, 1972). Furthermore, changes in surfactant would not explain the development of the histopathology produced by paraquat (Conning *et al.* 1969; Kimbrough & Gaines, 1970). It has been suggested that paraquat acts by initiating lipid peroxidation and thereby causing cell damage (Bus, Aust & Gibson, 1974), but Ilett, Stripp, Menard, Reid & Gillette (1974) considered that lipid peroxidation was not involved in the initiation of paraquat toxicity.

To characterize the paraquat lesion further, we have determined the relative subcellular distribution of paraquat and its effect on protein, DNA and RNA biosynthesis in the lung, liver and kidney of the rat. Biochemical changes were correlated, in time, with the clinical syndrome.

EXPERIMENTAL

Materials. Unlabelled paraquat (1,1′-dimethyl-4,4′-bipyridilium dichloride) was commercial-grade material supplied by Chevron Chemical Co., Richmond, Cal. The labelled compounds used were [^{14}C]paraquat (bis-(*N*-[^{14}C]methyl)-4,4′-bipyridilium chloride, from Amersham/Searle Corp., Arlington Heights, Ill.) and L-[U-^{14}C]leucine, [methyl-^{3}H]thymidine and [5-^{3}H]uridine (all from International Chemical and Nuclear Corp., Irvine, Ill.). Hyamine hydroxide was purchased from Packard Instrument Co., Inc., Downers Grove, Ill., and PCS from Amersham/Searle Corp.

Animals. Male Sprague–Dawley rats (from Spartan Research Animals, Inc., Haslett, Mich.) weighing 150–200 g were used in all experiments. The animals were housed in plastic cages in groups of three and were allowed food and water *ad lib.* Room temperature was maintained at 21–24°C and the light–dark cycle was 12 hr.

Subcellular distribution of [^{14}C]*paraquat.* Groups of rats were given ^{14}C-labelled paraquat (specific activity, 36 mCi/mmole) by gavage in a dose of 126 mg/kg (7-day LD_{50}) and 100 μCi/kg. At various times afterwards, groups of three animals were decapitated and the lungs, liver and kidneys were perfused with 10 ml 0·9% saline, removed, weighed and homogenized with a motor-driven teflon pestle in 3 vols buffer solution (0·25 M-sucrose, 0·25 M-KCl, 0·005 M-$MgCl_2$ and 0·05 M-Tris HCl, pH 7·5 at 20°C). The nuclear fraction from each tissue homogenate was isolated by centrifugation at 1000**g** for 10 min, the mitochondrial fraction was obtained from the nuclear supernatant by centrifugation at 10,000**g** for 30 min and the microsomal fraction was the pellet obtained from the mitochondrial supernatant by centrifugation at 100,000 **g** for 60 min. The soluble fraction was the resulting supernatant. The nuclear, mitochondrial and microsomal pellets were solubilized in 0·3 N-NaOH (10 ml/g) and heated at 40°C overnight. Aliquots (1 ml) of all four fractions were then placed in vials with 1 ml hyamine hydroxide and 10 ml PCS for liquid scintillation counting.

Precursor-incorporation study. Groups of rats received either 126 mg paraquat/kg or physiological saline by oral gavage. At various times afterwards, L-[^{14}C]leucine (specific activity 210 mCi/mmole; 100 μCi/kg), [^{3}H]thymidine (specific activity 14·1 Ci/mmole; 200 μCi/kg) or [^{3}H]uridine (specific activity 21–25 Ci/mmole; 400 μCi/kg) was administered

ip to groups of three rats. After a further hour, the animals were killed by decapitation and the lungs, liver and kidneys were perfused with 10 ml 0·9% saline, removed and weighed. The organs were homogenized in 0·2 N-perchloric acid (PCA; 0·5 g/5 ml), using a Polytron blender (Brinkmann Instruments, Westbury, New York). After centrifugation, the precipitate was washed twice with 5 ml portions of 0·2 N-PCA.

[^{14}C]Leucine incorporation into protein. The amount of [^{14}C]leucine incorporated into protein was determined by heating the tissue precipitate in 5 ml 0·6 N-PCA at 70°C for 30 min, followed by successive 5 ml washes with 95% ethyl alcohol saturated with sodium acetate, a 3:1 (v/v) ethanol–ether solution and ether to remove lipids. The final precipitate was dissolved in 10 ml 0·3 N-NaOH and heated at 40°C for 16 hr. Hyamine hydroxide (1 ml) and PCS (10 ml) were added to 0·5 ml aliquots of the protein solution for liquid scintillation counting. Protein concentration was determined by the Lowry procedure (Lowry, Rosebrough, Farr & Randall, 1951).

[^{3}H]Thymidine incorporation into DNA and [^{3}H]uridine incorporation into RNA. Lipid-extracted precipitates were heated at 37°C for 1 hr in 4 ml 0·3 N-NaOH, neutralized with 3 ml 30% trichloracetic acid (TCA), stored at 4°C for 16 hr and washed twice with 5 ml 5% TCA. In thymidine-incorporation studies, precipitates were heated twice in a boiling-water bath for 15 min in 5 ml 5% TCA and the resulting supernatants were combined and saved. PCS (10 ml) was added to 1 ml aliquots of the supernatants for liquid scintillation counting. The total DNA concentration was measured with diphenylamine (Burton, 1956).

In uridine-incorporation studies, lipid-extracted precipitates were heated at 37°C for 1 hr in 4 ml 0·3 N-NaOH, neutralized with 3 ml 30% TCA and stored at 4°C for 16 hr. The supernatant was saved and combined with that of an additional 2·5 ml wash with 10% TCA. PCS (10 ml) was added to aliquots of 1 ml of supernatant for liquid scintillation counting. RNA concentration was determined by the orcinol procedure (Ceriotti, 1955).

Statistical analyses were by analysis of variance and Student's t test. The level of significance was chosen as $P < 0{\cdot}05$.

RESULTS

All subcellular fractions of lung, liver and kidney contained [^{14}C]paraquat at all the times studied (Fig. 1) and the amount of paraquat/g subcellular tissue showed that the herbicide does not accumulate preferentially in any subcellular fraction. The subcellular distribution remained relatively constant in all three organs over time except in lung tissue where there was an apparent redistribution of paraquat to the microsomal fraction 32 hr after administration.

Protein, DNA and RNA biosynthesis (as indicated by the precursor incorporation rate) was not significantly affected in lung, liver or kidney for the first 8 hr after paraquat administration. However, 32 hr after paraquat administration, [^{14}C]leucine incorporation into protein was significantly increased in all three organs (Fig. 2). In the lung, the rate of protein synthesis remained high and was significantly elevated at 128 hr. After a peak at 32 hr the rate of protein synthesis in the liver gradually declined toward control values while, in the kidney, protein synthesis approached control levels more rapidly.

[^{3}H]Thymidine incorporation into DNA was significantly lower than control values in all three organs 32 hr after paraquat administration (Fig. 2) indicating a paraquat-related decrease in DNA synthesis. In the kidney, the decrease in DNA synthesis was significant at 64 hr and the rate remained low at 128 hr. By 64 hr, the rate of DNA synthesis in the liver and lung was approaching control values. The large standard error noted for lung

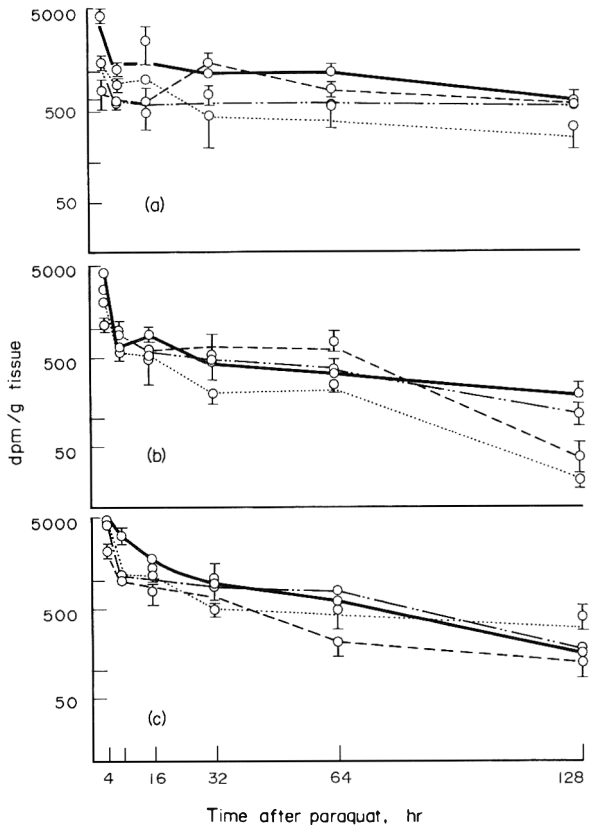

Fig. 1. Distribution of [^{14}C]paraquat in the soluble (——), microsomal (– – –), mitochondrial (·····) and nuclear (–·–) fractions of (a) lung, (b) liver and (c) kidney tissue at various times after administration of 126 mg paraquat/kg by gavage to rats. Each point is the mean of at least three determinations, the standard error being shown by vertical bars.

DNA synthesis 128 hr after paraquat administration may be a function of the 7-day LD_{50} dosage level selected, with the variation in DNA synthesis rates between minimally affected and severely affected animals being quite large. In fact, many severely affected animals were near death at this time.

The rate of uridine incorporation into RNA was significantly lower than the control rate in the liver and kidney 16 hr after paraquat treatment. After this time RNA synthesis in the liver steadily increased, while that in the kidney approached and remained near control levels. Although RNA synthesis in the lung was not significantly altered by paraquat, there was a trend toward an increased rate of uridine incorporation. This rate increased for 64 hr but returned to control levels by 128 hr.

DISCUSSION

The study described here demonstrated that paraquat penetrated the cells of the lung, liver and kidney and was distributed throughout the major cellular fractions, thus confirming the observations of Ilett *et al.* (1974). No evidence of significant redistribution of paraquat from one subcellular fraction to another was found during 128 hr after treatment

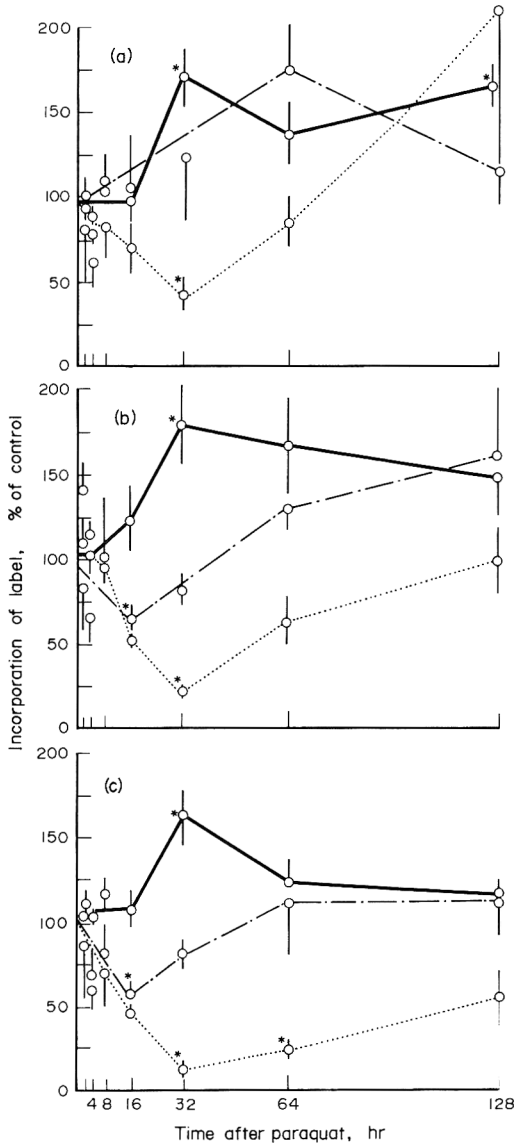

Fig. 2. DNA (····), RNA (– · –) and protein (——) biosynthesis in (a) lung, (b) liver and (c) kidney tissue at various times after administration of 126 mg paraquat/mg to rats by gavage. Each point represents the mean of at least three determinations with the standard error shown by vertical bars. Points which vary significantly ($P < 0{\cdot}05$) from control values are indicated by an asterisk.

except in the lung, where 32 hr after administration the microsomal fraction appeared to accumulate paraquat. This observation may be related to the whole-lung accumulation of paraquat in the rat noted in previous studies (Murray & Gibson, 1974; Sharp *et al.* 1972), in which the peak paraquat concentrations were reached 32 hr after administration of an oral 7-day LD_{50} dosage.

In this study, the earliest biochemical changes noted in the rat lung were also at 32 hr. Thus, the increased protein synthesis and decreased DNA synthesis correlated with the

peak concentration of paraquat in the lungs. However, this correlation was not true for the liver and kidney, as these organs had their highest concentration of paraquat during the first 4 hr after administration of the herbicide (Murray & Gibson, 1974). The fact that no biochemical changes were noted in the lungs earlier than 32 hr after paraquat administration agreed with the results of Witschi (1973), who detected no significant change in RNA or protein synthesis during the first 24 hr after paraquat treatment.

Early histological changes in the lungs of paraquat-treated rats included increased collagen within the intercellular spaces, "ribosome-like" granules becoming larger and more prominent in the cytoplasm of membranes (Type I) pneumocytes, and advanced stages of swelling and degeneration of these alveolar lining cells (Kimbrough & Gaines, 1970; Kimbrough & Linder, 1973; Modée, Ivemark & Robertson, 1972; Neinhaus & Ehrenfeld, 1971). These findings were observed as early as 2 days after paraquat administration (Kimbrough & Gaines, 1970). The increase in protein synthesis noted in this study correlated with the proliferation of endoplasmic reticulum and the enlargement of "ribosomal" granules. In addition, the decrease in DNA synthesis appeared consistent with the presence of degenerated and vacuolated cells. Thus, the biochemical changes 32 hr after paraquat administration coincided with the appearance of the histopathological effects and both preceded the clinical signs of dyspnoea and hyperpnea, which occurred in rats 3–4 days after treatment with paraquat.

In the kidneys of paraquat-poisoned mice, vacuolated proximal tubular cells and proliferation of smooth endoplasmic reticulum were observed by electron microscopy (Fowler & Brooks, 1971). The increase in endoplasmic reticulum noted in mice may also occur in the rat and be related to the increased protein synthesis observed in the present study, while the presence of vacuolated cells suggests decreased DNA and RNA synthesis. Clinically, oliguria, albuminuria and increased blood urea nitrogen were often seen in acute paraquat poisoning in man (Fisher, Humphries & Bails, 1971; McDonagh & Martin, 1970; Pasi & Hine, 1971). Therefore, the histological and biochemical findings were consistent with the clinical signs and symptoms of kidney damage.

In the liver of paraquat-poisoned monkeys, areas of centrilobular necrosis were observed microscopically, but serum glutamic–oxalacetic and glutamic–pyruvic transaminases revealed no definite hepatic dysfunction (Murray & Gibson, 1972). One child poisoned with the herbicide displayed centrilobular stasis and became jaundiced (McDonagh & Martin, 1970). The decrease in DNA and RNA synthesis noted in our study therefore correlated with necrosis of hepatic cells and, in at least one instance, with the clinical syndrome.

Whether paraquat induces toxic effects by direct inhibition of DNA and RNA synthesis and increases protein synthesis at some cellular control level is uncertain. In other studies with rats, paraquat increased blood glucose, plasma corticosteroids and the response of the adrenal cortex to ACTH (Rose, Crabtree, Fletcher & Wyatt, 1974). However, those biochemical findings, like the present observations on DNA, RNA and protein, could reflect changes secondary to the actions of paraquat rather than a direct effect.

In plants, the phytotoxic action of paraquat is assumed to be due to the reaction between the paraquat radical and oxygen, resulting in the successive formation of the superoxide ion (O_2^-), hydrogen peroxide (H_2O_2) and the hydroxy radical (OH^-) (Calderbank, 1968; Farrington, Ebert, Land & Fletcher, 1973). Reactive free radicals may exert toxic effects on biological systems by oxidizing sulphydryl groups (Robinson, 1966), or by initiating polymerization of unsaturated lipid constituents or the peroxidation of unsatur-

ated lipids (Pryor, 1973). An increase in lipid-peroxide formation found in paraquat-treated plants was consistent with free radical pathology (Baldwin, Dodge & Harris, 1968).

Animal tissues are capable of reducing paraquat under anaerobic conditions to a stable free radical (Bus *et al.* 1974; Gage, 1968). Furthermore, oxygen enhances paraquat toxicity in rats (Fisher, Clements & Wright, 1973), suggesting the possibility of a sequence of reactions between paraquat and oxygen in animals as in plants. Since superoxide dismutase (Autor, 1974) protects against paraquat toxicity, an interaction with oxygen is possible. Bus *et al.* (1974) reported a paraquat-induced increase in the *in vitro* peroxidation of rat-liver microsomal lipid and suggested that paraquat might initiate peroxidation of unsaturated lipids. The proposed mechanism involved the transfer of an electron from reduced paraquat to oxygen with the subsequently generated superoxide anion as the toxic species. However, Ilett *et al.* (1974) were unable to demonstrate paraquat initiation of lipid peroxidation *in vitro*.

The changes in the synthesis of cellular protein and nucleic acid components in paraquat-poisoned animals may be secondary to paraquat-initiated free-radical toxicity rather than the direct result of paraquat action on cellular control mechanisms.

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MUTAGENICITY STUDIES WITH CAPTAN, CAPTOFOL, FOLPET AND THALIDOMIDE

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Abstract—The three fungicides, captan, captofol and folpet and a structurally related compound, thalidomide, were investigated for possible mutagenic activity by the dominant lethal mutagenicity study in mice and by the host-mediated assay in rats using a histidine auxotroph of *Salmonella typhimurium*. Male mice were treated with a single ip injection of captan (3 or 6 mg/kg), captofol (1·5 or 3·0 mg/kg), folpet (5 or 10 mg/kg) or thalidomide (500 or 1000 mg/kg). The results of weekly matings with untreated virgin females disclosed no increase in early embryonic death among conceptuses obtained from females mated with the treated males. Groups of male rats were treated by oral intubation for 14 days with one of the three fungicides at levels of 125 or 250 mg/kg/day or with thalidomide at 500 or 1000 mg/kg/day. Indicator micro-organisms recovered from the peritoneal cavity of the treated male rats after a 3-hr residence showed no increase in numbers of revertants. Thus captan, captofol, folpet and thalidomide were not mutagenic in either of these test systems.

INTRODUCTION

Investigators have studied the possible teratogenic and mutagenic properties of the fungicides captan (*N*-trichloromethylthiotetrahydrophthalimide), captofol (Difolatan®; *N*-1,1,2,2-tetrachloroethylthiotetrahydrophthalimide) and folpet (Phaltan; *N*-trichloromethylthiophthalimide). Mammalian, avian and bacterial systems have been used in these studies but findings have been inconsistent.

Legator, Kelly, Green & Oswald (1969) found that captan levels of 1000 μg/12·7 mm assay disc on 15 ml of seeded medium caused increased mutation rates in streptomycin-dependent and thymine-dependent strains of *Escherichia coli*. In human embryonic lung cells (L-132), captan levels of less than 5 μg/ml inhibited growth and mitosis, but these inhibitions were generally overcome after 48 hr. Finally, in a rat kangaroo cell line, captan induced breaks in the centromeric region of the X chromosome. However, using mice in the host-mediated assay and in an *in vitro* study, both with a histidine auxotroph of *Salmonella typhimurium*, reversion to prototrophs in response to exposure to captan was not observed (Legator & Malling, 1971). Other studies with captan included a spot test with a repair-deficient strain of *E. coli* (Bridges, Mottershead, Rothwell & Green, 1972), an *in vitro* and *in vivo* system using *S. typhimurium* (his G46), and a host-mediated assay with a leucine-requiring strain of *Serratia marcescens* and mice (Buselmaier, Rohrborn & Propping, 1972); these gave strongly positive results, leading Bridges *et al.* (1972) to suggest that inhaled captan might be potentially carcinogenic (as it would dissolve very slowly in the lung).

Verrett, Mutchler, Scott, Reynaldo & McLaughlin (1969) reported that all three fungicides caused phocomelic and amelic abnormalities in chick embryos. McLaughlin,

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Reynaldo, Lamar & Marliac (1969) obtained abnormal young, with malformations including limb deformities, cleft lip and acephelia, from captan-treated female rabbits, but young from folpet-treated females were normal. Fabro, Smith & Williams, (1965). Kennedy, Fancher & Calandra (1968) and Vondruska, Fancher & Calandra (1971) reported studies with rabbits, rats, hamsters and non-human primates and found no embryotoxic response after treatment during organogenesis with any of the three fungicides.

On the basis of data obtained in dominant lethal studies in rats and mice, Collins (1972a, b) suggested that there was no mutagenic danger to man from the levels of intake resulting from the current tolerances for these compounds. However, in a reproduction study in mice (Collins, 1972c), he interpreted minor differences in reproductive parameters as indicating that genetic effects from captan might have been occurring in this species. In dominant lethal studies of many compounds, Epstein, Arnold, Andrea, Bass & Bishop (1972) found no indication of induced mutations among captan-treated male mice.

Kramers & Knaap (1973), using the induction of sex-linked lethals in Drosophila, observed no demonstrable mutagenic effect of captan or folpet, but did not exclude the possibility of a very mild effect. The studies reported here are attempts to relate treatment with these compounds to possible mutagenic changes in mammalian test systems.

EXPERIMENTAL

Materials and animals. Captan, captofol and folpet were supplied by Chevron Chemical Co., Richmond, Cal., and dimethylnitrosamine by Eastman Kodak, Rochester, N.Y. Thalidomide was synthesized in these laboratories. Albino mice of the Charles River strain ranging in age from 60 to 70 days were used for the dominant lethal study, while the host-mediated assay was conducted in Charles River male albino rats weighing 250–300 g, using a histidine-dependent strain of *S. typhimurium*, his G46.

Dominant lethal study. Two dose levels were selected for each compound on the basis of preliminary acute toxicity studies. Groups of mice were given captan (3 or 6 mg/kg), captofol (1·5 or 3·0 mg/kg), folpet (5 or 10 mg/kg) or thalidomide (500 or 1000 mg/kg) in single ip doses in corn oil. Additionally, three separate control groups, all treated with corn oil, were used. Each test and control group consisted of 12 male mice, each of which was dosed and immediately housed with three untreated virgin females. The females were removed weekly, transferred to holding cages and replaced by another three untreated virgin females. This procedure was continued for six consecutive weeks, which is the normal time for spermatogenic maturation in the mouse (Oakberg, 1960). Females were killed by carbon dioxide asphyxiation 1 wk after their removal from the breeding cage, at which time most of those that had mated were at mid-pregnancy. The numbers of implantation sites, consisting of resorption sites and/or embryos, were recorded. Resorption sites were divided into two groups, covering those resulting from early deaths (deciduomas) and those from late embryonic deaths. Since late deaths are apparently not genetically induced (Bateman, 1966), they were disregarded in the calculation of mutation rates, which were estimated on the basis of the following parameters:

$$\text{Pre-implantation loss} = \frac{\text{Total no. of corpora lutea (calc.)} - \text{Total implantation sites}}{\text{Total no. of corpora lutea (calc.)}} \times 100$$

$$\text{Percentage of deciduomas} = \frac{\text{No. of deciduomas (early embryonic deaths)}}{\text{Total implantation sites}} \times 100$$

$$\text{Embryonic index} = \frac{\text{No. of embryos/control females} - \text{No. of embryos/test females}}{\text{No. of embryos/control females}} \times 100$$

Host-mediated assay. For the host-mediated assay (Gabridge & Legator, 1969), two separate trials were conducted for each fungicide, using three males in each of two test groups, one negative control group and one positive control group. Treatment was continued for 14 days and consisted of daily oral intubation of captan, captofol or folpet at levels of 125 or 250 mg/kg or 500 or 1000 mg thalidomide/kg. Compounds were mixed in corn oil. After the dosing period the rats were given ip 5 ml of a broth culture of the Salmonella containing approximately $1{\cdot}25 \times 10^8$ organisms/ml. Simultaneously with the bacterial inoculation, positive control animals received dimethylnitrosamine (DMN) in an intramuscular dose of 100 mg/kg.

The animals were killed 3 hr after treatment and peritoneal fluid was recovered aseptically. Serial dilutions to 10^{-6} of the peritoneal washings were prepared in sterile, normal saline. A 0·2 ml aliquot of the diluted peritoneal fluid was added to 1·5 ml of sterile agar containing histidine; a 0·4 ml aliquot of the undiluted peritoneal fluid was added to 1·5 ml of sterile agar without histidine and each preparation was overlayed on sterile agar in petri dishes in duplicate. After a 48-hr incubation, colony counts on each plate were recorded and mutation rates were calculated as ratios of prototrophs (growth on histidine-deficient medium) to total organisms (growth on histidine-supplemented medium).

RESULTS AND DISCUSSION

Dominant lethal study

One male given 3 mg captofol/kg died after 2 wk of testing and one thalidomide-treated male (1000 mg/kg) died at the end of wk 1 of testing. No other deaths occurred. Folpet-treated males (5 and 10 mg/kg) had ruffled fur, while the treated males at the 10 mg/kg level were also slightly hypoactive. Thalidomide-treated males (500 and 1000 mg/kg) were somewhat hypoactive for 1–2 days after treatment. The percentages of pregnant females (Table 1) for animals treated with 10 mg folpet/kg were somewhat low at wk 4, 5 and 6. However, these are not considered unusual for the mouse strain used. Indices for all other groups and weeks compared favourably with those of controls.

Data presented in Table 1 pertaining to the numbers of implantation sites, resorption sites (early and late) and embryos were essentially the same for treated animals as for the control matings. The numbers of corpora lutea were calculated by multiplying the number of pregnant females by 13·5, historically the mean numbers of corpora lutea for untreated females obtained at the laboratory. This was done because of the inherent difficulty in counting corpora lutea in mice and was used to estimate the incidence of pre-implantation loss. Mutation rates, defined as the number of deciduomas divided by the total number of implantation sites multiplied by 100, were normal for all the groups of fungicide-treated animals and compared favourably with the mutation rates for control animals (Table 2). The embryonic index, which also reflects pre-implantation losses, can be altered through non-genetic means, and should therefore be used mainly as a guide in the evaluation of induction of dominant lethals.

Host-mediated assay

No increases over the spontaneous control rate were obtained in the numbers of revertants (mutants) to prototrophic type among the bacteria recovered from the peritoneal cavity of treated rats and plated on selective media (Table 3). Revertant rates for the treated groups were well within the normal range of values obtained for untreated animals.

Table 1. *Reproductive performance in untreated female mice mated with males treated ip with captan, captofol, folpet or thalidomide (dominant lethal study)*

Group	Dose level (mg/kg)	Test wk no.	Females pregnant (%)	Total no. of		Mean no. of embryos pregnancy
				Implantations	Deciduomas	
Control*		1	71·3	891	39	11·0
		2	73·1	966	47	11·6
		3	60·2	790	43	11·3
		4	79·6	1045	49	11·5
		5	67·6	904	44	11·7
		6	66·7	850	35	11·2
Captan	3	1	69·4	282	15	10·6
		2	86·1	347	26	10·3
		3	77·8	335	13	11·1
		4	83·3	372	14	11·8
		5	91·7	364	14	10·6
		6	72·2	327	20	11·7
	6	1	66·7	263	19	10·1
		2	69·4	311	25	11·4
		3	66·7	285	8	11·5
		4	80·6	364	15	11·9
		5	72·2	314	13	11·4
		6	88·9	403	27	11·6
Captofol	1·5	1	69·4	309	12	11·8
		2	75·0	320	18	11·0
		3	83·3	351	10	11·3
		4	88·9	415	14	12·4
		5	86·1	360	19	11·0
		6	77·8	338	23	11·0
	3	1	69·4	300	18	11·1
		2	63·9	295	25	11·6
		3	63·6	238	5	11·0
		4	69·7	261	7	10·9
		5	60·6	215	16	10·0
		6	66·7	252	11	10·8
Folpet	5	1	68·6	282	17	11·0
		2	57·1	248	21	11·2
		3	69·4	316	27	11·4
		4	55·6	259	17	11·6
		5	52·8	243	12	12·0
		6	68·6	305	31	11·2
	10	1	58·3	255	7	11·5
		2	50·0	219	9	12·1
		3	67·6	300	14	12·3
		4	48·3	195	12	12·6
		5	48·5	208	17	11·6
		6	43·8	189	12	11·6
Thalidomide	500	1	69·4	328	19	12·4
		2	77·8	329	15	11·2
		3	75·0	327	15	11·5
		4	91·7	386	24	11·0
		5	63·9	261	9	10·9
		6	61·1	276	14	11·9
	1000	1	50·0	220	16	11·3
		2	54·5	217	12	11·4
		3	72·7	282	7	11·4
		4	63·6	235	16	10·3
		5	69·7	274	9	11·5
		6	69·7	292	14	12·0

* Values derived from three separately-run control groups.

Table 2. *Mutation rates in untreated female mice mated with males treated ip with captan, captofol, folpet or thalidomide (dominant lethal study)*

Group	Dose level (mg/kg)	Test wk no.	Pre-implantation loss (%)	Deciduomas (%)	Embryonic index
Control*		1	14·3	4·4	--
		2	9·4	4·9	–
		3	10·0	5·4	–
		4	10·0	4·7	
		5	8·3	4·9	–
		6	12·6	4·1	
Captan	3	1	16·6	5·3	3·6
		2	17·0	7·5	11·2
		3	11·4	3·9	1·8
		4	8·1	3·8	−2·6
		5	18·4	3·8	9·4
		6	6·8	6·1	−4·5
	6	1	18·8	7·2	8·2
		2	8·0	8·0	1·7
		3	12·0	2·8	−1·8
		4	7·1	4·8	−3·5
		5	10·5	4·1	2·6
		6	6·7	6·7	−3·6
Captofol	1·5	1	8·6	3·9	−7·3
		2	12·1	5·6	5·2
		3	13·3	2·8	0·0
		4	3·9	3·4	−7·8
		5	13·9	5·3	6·0
		6	10·6	6·8	1·8
	3	1	11·2	6·0	−0·9
		2	4·8	8·5	0·0
		3	16·2	2·1	2·7
		4	15·8	2·7	5·2
		5	20·4	7·4	14·5
		6	15·2	2·4	3·6
Folpet	5	1	13·0	6·0	0·0
		2	8·1	8·5	3·4
		3	6·5	8·5	−0·9
		4	4·1	6·6	−0·9
		5	5·1	4·9	−2·6
		6	5·9	10·2	0·0
	10	1	10·2	2·7	−4·5
		2	4·8	4·1	−4·3
		3	3·2	4·7	−8·8
		4	0·0	6·2	−9·6
		5	3·7	8·2	0·9
		6	0·0	6·3	−3·6
Thalidomide	500	1	3·0	5·8	−12·7
		2	13·0	4·6	3·4
		3	10·2	4·6	−1·8
		4	13·4	6·2	4·4
		5	15·8	3·4	6·8
		6	7·1	5·1	−6·2
	1000	1	9·5	7·3	−2·7
		2	10·7	5·5	1·7
		3	13·0	2·5	−0·9
		4	17·2	6·8	10·4
		5	11·6	3·3	1·7
		6	5·8	4·8	−7·1

* Values derived from three separately-run control groups.

Table 3. *Mean revertant (mutation) rates in* Salmonella typhimurium *used as the indicator micro-organism in albino rats intubated daily for 14 days with captan, captofol, folpet or thalidomide (host-mediated assay)*

Group	Dose level (mg/kg/day)	Revertant rate* in trial no. 1	2
Control†	0	2·50	1·56
Dimethylnitrosamine†	100	11·71	12·35
Captan	125	0·42	1·68
	250	3·06	1·08
Captofol	125	1·91	1·78
	250	1·73	1·56
Folpet	125	2·24	1·63
	250	2·07	1·59
Thalidomide	500	1·91	1·34
	1000	2·89	1·82

*(Number of prototrophs/ml/Number of organisms/ml) × 100.
†Values derived from four separately-run control groups.

DMN-treated animals had revertant rates some 5–10 times higher than the spontaneous rates.

Conclusions

The results of these experiments indicate that captan, captofol and folpet were not mutagenic in either of the two test systems. The findings reported here and elsewhere point to the need to use mammalian test systems in the detection of mutagenic potential, and indicate that caution should be used in extrapolating to higher organisms effects observed in bacterial strains. In addition, it is clear that studies beyond the scope of the dominant lethal and host-mediated assays may be required for the full evaluation of the portended genetic hazard from captan and these related chemicals. Captan appears to have the ability to produce mutations in bacteria, but evidence for these changes in mammalian species is lacking.

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MUTAGENICITY STUDIES WITH δ-AMINOLAEVULINIC ACID

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Abstract—Groups of eight male albino mice were treated by gavage or ip injection with δ-aminolaevulinic acid at dose levels of 0·25, 0·50 or 1·00 g/kg body weight. All doses were administered daily for 1 wk except for the ip dose of 1 g/kg, which was only given once because of toxicity. The males were each subsequently mated with three females/wk for six consecutive weeks. No genetic damage, manifested as dominant lethal mutations observed *in utero*, was evident in these females.

INTRODUCTION

δ-Aminolaevulinic acid (δ-ALA) and porphobilinogen are important constituents in the production of both porphyrin and heme (Falk, Dresel & Rimington, 1953; Shemin & Russell, 1953). It has been suggested that a deficiency in δ-ALA dehydrase, the enzyme responsible for catalysing porphobilinogen from δ-ALA, may be significant in certain hereditary anaemias (Feldman & Lichtman, 1962). Increased blood-lead levels have been associated with a reduction of erythrocyte δ-ALA dehydrase (Haeger-Aronsen, Abdulla & Fristedt, 1971), while Weissberg, Lipschutz & Osaki (1971) have found this to be an inverse correlation.

In lead poisoning, δ-ALA among other metabolites accumulates in the plasma and red blood cells (Bonsignore, 1966). The same investigator also found that this occurred despite the fact that the activity of δ-ALA dehydrase is severely lessened in human erythrocytes in lead poisoning. Potential genetic damage by increased δ-ALA levels was investigated using the induction of dominant lethal mutations as the criterion.

EXPERIMENTAL

Materials. δ-ALA (δ-aminolaevulinic acid HCl) was obtained from Nutritional Biochemicals Corp., Cleveland, Ohio.

Animals and diet. The animals used were CD-1 random-bred albino mice purchased from Charles River Breeding Laboratories, Wilmington, Mass. They were maintained on a standard pelleted feed, obtained from Ralston-Purina, St. Louis, Mo., and water provided *ad lib.*

Experimental design. Male mice were divided into eight groups each of eight animals and were treated either by oral intubation or ip injection with a 5% aqueous solution of δ-ALA at dose levels of 0·25, 0·50 or 1·00 g/kg body weight. Concurrent control animals, one group for each route of administration, were treated with distilled water in volumes equivalent to those received by the mice given 1·0 g δ-ALA/kg. Animals were treated daily for 1 wk with the exception of the males given 1·00 g/kg ip, which were treated only once. Originally, these males were to be given the same length of treatment as the other groups,

but five of the eight died before the fifth dose, so the number of treatments was reduced to one.

Immediately after the final dose, each male was housed individually with three untreated nulliparous females. The females were changed weekly, caged by group and replaced with fresh females for a period of six consecutive weeks. The females were killed 1 wk after their removal from the breeding cages and were examined for signs of pregnancy. The numbers of implantation sites were divided into embryos and resorption sites which were differentiated as early (deciduoma) and late. From these data, mating indices and mutation rates were calculated according to the following definitions:

$$\text{Mating index} = \frac{\text{No. of females pregnant}}{\text{No. of females mated}} \times 100$$

$$\text{Pre-implantation loss} = \frac{\text{No. of corpora lutea (calc.)} - \text{No. of implantation sites}}{\text{No. of corpora lutea (calc.)}} \times 100$$

$$\text{Percentage of deciduomas} = \frac{\text{No. of early embryonic deaths (deciduomas)}}{\text{Total no. of implantation sites}} \times 100$$

$$\text{Embryonic index} = 100 - \left(\frac{\text{No. of embryos/female test}}{\text{No. of embryos/female control}} \times 100\right)$$

In calculating the total number of corpora lutea, the 'historical number' of corpora lutea/female was used. This value was obtained from corpora-luteal counts obtained from untreated male–female pairing and histological examination of ovaries from pregnant females. This procedure was used to overcome the difficulty inherent in counting corpora lutea accurately in mice and to allow an estimation of pre-implantation losses in these animals.

RESULTS AND DISCUSSION

The autopsies on females that had mated (Tables 1 & 2) revealed no differences between test and control groups in the numbers of implantations, resorptions (deciduomas) and embryos. The mating abilities of treated animals (Tables 3 & 4) were generally comparable with those of the control groups, with the exception of the males given 1·0 g/kg ip. in which group fertility was slightly reduced for the first 2 wk following treatment. In view of the fact that all other values for this group were normal, the lowered mating ability appeared to be more of a toxic than genetic effect. The normal mating indices in control populations for this strain of mouse range from 50 to 90% and most values obtained in this study fell within this range.

Mutation rates (Tables 3 & 4), calculated from the data obtained at autopsy, were essentially the same for treated animals as for controls. The embryonic index is an indirect measure of pre-implantation losses. These losses, however, can also arise through non-genetic means, such as sperm toxicity when fertilization fails to occur, and therefore the index should serve as a guide rather than as an exact measure of induced dominant lethality. Mutagenicity of δ-ALA, as demonstrated by early embryonic death, was not evident in this test system.

Poisoning by heavy metals may cause increases in blood δ-ALA. Aside from the direct damage caused by heavy-metal poisoning, the increase in δ-ALA levels does not appear to stimulate genetic activity.

Table 1. *Mean values for reproduction data in untreated female mice mated with males treated by oral intubation with 0–1·00 g δ-ALA/kg/day for 1 wk*

Dose level (g/kg/day)	Test wk no.	No. of females pregnant	Implantation sites*	Resorption sites*		Embryos*
				Early	Late	
0	1	14	12·1	0·4	0·0	11·8
	2	14	11·8	0·3	0·1	11·4
	3	18	11·4	0·6	0·1	10·8
	4	20	11·0	0·9	0·0	10·1
	5	15	11·7	0·6	0·1	10·9
	6	15	12·1	0·6	0·0	11·5
0·25	1	20	11·2	0·5	0·0	10·8
	2	19	11·6	0·3	0·1	11·3
	3	20	11·6	1·0	0·1	10·6
	4	19	12·1	0·5	0·1	11·5
	5	18	11·9	0·5	0·1	11·3
	6	12	12·7	0·2	0·1	12·3
0·50	1	11	11·6	0·3	0·0	11·4
	2	19	11·1	0·4	0·2	10·6
	3	16	11·9	0·4	0·1	11·4
	4	20	12·2	0·7	0·1	11·5
	5	20	11·2	0·4	0·0	10·7
	6	19	13·1	0·6	0·1	12·5
1·00	1	14	12·1	0·2	0·0	11·9
	2	16	11·0	0·3	0·0	10·7
	3	19	12·0	0·7	0·1	11·3
	4	18	11·7	0·5	0·1	11·2
	5	20	10·8	0·6	0·1	10·0
	6	17	12·9	0·7	0·2	12·0

* Mean no./pregnancy.

Table 2. *Mean values for reproduction data in untreated females mated with males given ip injections of δ-ALA in doses of 0–1·00 g/kg for 1 or 7 days*

Dose level (g/kg/day)*	Test wk no.	No. of females pregnant	Implantation sites†	Resorption sites†		Embryos†
				Early	Late	
0	1	18	11·9	0·5	0·0	11·4
	2	18	11·2	0·3	0·1	10·9
	3	16	12·1	0·6	0·0	11·4
	4	16	13·0	0·4	0·1	12·4
	5	16	10·5	0·9	0·0	9·6
	6	16	11·8	0·6	0·0	11·1
0·25	1	17	12·1	0·6	0·0	11·5
	2	22	11·4	0·4	0·1	11·0
	3	19	12·2	0·8	0·1	11·3
	4	19	12·5	0·8	0·1	11·6
	5	19	11·5	0·6	0·1	10·7
	6	20	12·2	1·3	0·1	10·8
0·50	1	17	10·8	0·4	0·0	10·5
	2	15	11·6	0·1	0·1	11·5
	3	14	13·2	0·6	0·1	12·4
	4	13	12·0	0·7	0·0	11·3
	5	9	11·6	0·7	0·0	10·9
	6	12	10·8	0·8	0·0	9·9
1·00	1	9	10·9	0·2	0·0	10·7
	2	9	13·0	0·4	0·1	12·4
	3	14	12·6	0·1	0·1	12·4
	4	16	11·6	0·6	0·0	11·1
	5	15	12·5	0·6	0·0	11·7
	6	17	12·8	0·7	0·1	12·0

* Doses of 0, 0·25 and 0·50 g/kg given daily for 1 wk; 1·00 g/kg given in a single dose.
† Mean no./pregnancy.

Table 3. *Mating indices and mutation rates in untreated female mice mated with males treated by oral intubation with 0–1·00 g δ-ALA/kg/day for 1 wk*

Dose level (g/kg/day)	Test wk no.	Mating index	Pre-implantation loss (%)	Deciduomas (%)	Embryonic index
0	1	58·3	10·0	2·9	—
	2	58·3	12·7	2·4	—
	3	75·0	15·2	4·8	—
	4	83·3	18·5	8·2	—
	5	62·5	13·4	4·1	—
	6	62·5	10·4	5·0	—
0·25	1	83·3	16·7	4·4	8·5
	2	79·2	13·7	2·7	0·9
	3	83·3	13·7	8·6	1·9
	4	79·2	10·2	4·3	−13·9
	5	75·0	11·5	4·2	−3·7
	6	50·0	6·2	2·0	−7·0
0·50	1	45·8	13·5	2·3	3·7
	2	79·2	17·6	3·3	7·0
	3	66·7	12·0	3·7	−5·6
	4	83·3	9·2	5·7	−13·9
	5	83·3	17·4	4·0	1·8
	6	79·2	2·7	4·0	−8·7
1·00	1	58·3	10·0	1·8	−0·8
	2	66·7	18·5	2·8	6·2
	3	79·2	10·5	5·7	−4·6
	4	75·0	13·2	4·3	−10·9
	5	83·3	20·4	6·0	8·3
	6	70·8	4·8	5·5	−4·3

Table 4. *Mating indices and mutation rates in untreated female mice mated with males given ip injections of δ-ALA in doses of 0–1·00 g/kg for 1 or 7 days*

Dose level (g/kg/day)*	Test wk no.	Mating index	Pre-implantation loss (%)	Deciduomas (%)	Embryonic index
0	1	75·0	11·9	4·2	—
	2	75·0	16·9	2·5	—
	3	66·7	10·6	5·2	—
	4	66·7	3·7	3·4	—
	5	66·7	22·2	8·9	—
	6	66·7	13·0	5·3	—
0·25	1	70·8	10·4	5·3	−0·9
	2	91·7	15·2	4·0	−0·9
	3	79·2	9·8	6·9	0·9
	4	79·2	7·4	6·8	6·5
	5	79·2	14·8	5·5	−11·4
	6	83·3	9·6	10·6	2·7
0·50	1	70·8	20·0	3·3	9·9
	2	62·5	13·9	0·6	−5·5
	3	58·3	2·1	4·3	−8·8
	4	54·2	11·4	5·8	8·9
	5	37·5	14·8	5·8	−13·5
	6	50·0	20·4	7·8	10·8
1·00	1	37·5	19·7	2·0	6·2
	2	37·5	4·1	3·4	−13·8
	3	58·3	6·3	1·1	−8·8
	4	66·7	13·9	4·8	10·5
	5	62·5	7·9	4·8	−5·4
	6	70·8	5·2	5·5	−8·1

* Doses of 0, 0·25 and 0·50 g/kg given daily for 1 wk; 1·00 g/kg given in a single dose.

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EFFECTS OF HEXACHLOROPHENE ON DEVELOPING RATS: TOXICITY, TISSUE CONCENTRATIONS AND BIOCHEMISTRY*

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Abstract—Hexachlorophene (HCP) was administered to nursling rats by gavage at different periods of early development with very different results. Rats dosed with 10 mg HCP/kg/day beginning at 2–4 days of age developed signs of neurotoxicity at 9–10 days of age. These signs disappeared at 23–30 days of age even with continued dosing, and no deaths were attributable to HCP. If, however, dosing was begun at 10–13 days of age, all rats died after 2–3 doses. When administration began at 22–60 days of age there were no signs of neurotoxicity even with continued dosing to 87 days of age. Signs of neurotoxicity were accompanied by a diffuse vacuolation of the white matter throughout the brain, but most noticeably in the brain stem, in all rats examined for pathological changes. The concentrations of HCP in blood and brain were highest in 13-day-old rats and lower in adults and in 4–8-day-old rats, and there was no accumulation of HCP in brain, liver or adipose tissue. HCP appeared to reach a peak concentration in the blood at 3–6 hr after the last dose, the concentrations in the blood and liver being higher than those in the brain or fat. No gross alteration was induced by HCP in brain ATP, phospholipid, sterol, protein, DNA or RNA or in the incorporation of radioactive precursors into these molecules.

INTRODUCTION

Hexachlorophene (2,2′-methylenebis-(3,4,6-trichlorophenol; HCP), a well-known antimicrobial agent, has been investigated extensively in the last few years. The toxicity of HCP in animals and in man has been reviewed by Gump (1969), Kimbrough (1971) and Lockhart (1972). The acute oral LD_{50} of HCP in peanut oil was reported to be 56 mg/kg for adult female rats and 66 mg/kg for adult male rats (Gaines, Kimbrough & Linder, 1973).

Kimbrough & Gaines (1971) fed adult rats diets containing 500 ppm HCP. Nervousness and weakness of the hindquarters were observed after 2 wk and were followed by paralysis of the hindquarters in 3–5 wk. After the rats had been fed the diet for 14 wk, the wet weights of the brains had increased by 30% over those of controls. Microscopic examination revealed extensive vacuolation in the white matter of the brain and spinal cord (Kimbrough, 1973) as early as 2 wk after exposure to HCP (although the grey matter appeared to be normal). Termination of exposure to high dietary levels of HCP resulted in a gradual return of leg function and a reduction in morphological changes in the brain of adult female rats. The changes in brain morphology produced by HCP given at 100 ppm in the

*A preliminary report on part of this work was presented at the Annual Meeting of the Society of Toxicology held in Williamsburg, Va, in March 1972.

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diet were minimal, consisting of a few vacuoles with no visible signs of neurotoxicity (Kimbrough & Gaines, 1971). Curley & Hawk (1971) reported that the average of the HCP concentrations in the blood of four rats given a 100 ppm dietary level of HCP was 1·2 μg/ml, whereas in two rats fed 500 ppm it was 8·5 μg/ml in blood and 1·7 μg/g in brain tissue.

These earlier studies were concerned with the effects on adult rats of ingesting HCP. We decided, therefore, to examine the effects of HCP on nursling rats, since these animals have a developing nervous system and an incomplete blood–brain barrier (McIlwain, 1966). In addition, certain biochemical parameters were investigated in an effort to provide information on the mechanism of production of the CNS lesion. Our findings on young and adult rats dosed with HCP by gavage are described in the present paper.

EXPERIMENTAL

Animals. Osborne–Mendel rats of both sexes were obtained from a colony maintained at the laboratories of the Food and Drug Administration. The rats were kept in a light-controlled environment at 24° C, and food and water were provided *ad lib.* Body weights were recorded weekly.

Treatment. HCP was supplied by Givaudan Corp., Clifton, N.J. 07014, and a working solution of 2 mg/ml in corn oil was prepared every 2 wk and stored in a light-proof bottle at room temperature. The rats were dosed by gavage in a dose of 10 mg/kg/day on 5 days/wk (Monday to Friday) unless otherwise stated, and were killed on Thursday or Friday. Control rats received only corn oil.

Microscopy. Rats were killed by decapitation and the brains were removed and immediately immersed in buffered formalin. The tissue was then embedded, sectioned and stained with haematoxylin and eosin, as described by Kimbrough & Gaines (1971).

Water content of brain. Brains were removed and weighed. The brains were then desiccated to a constant weight under vacuum over P_2O_5. Water content was calculated from the difference between wet and dry weights.

HCP determinations. HCP was extracted from blood and tissues. After acetylation it was determined by gas–liquid chromatography according to the method of Ulsamer (1972), using a ^{63}Ni electron-capture detector. Recovery was quantitative when HCP was added to blood in a concentration of 5·0 μg/ml and 95% when 0·01 μg/ml was added; the range was 90–100% for all concentrations studied. Recovery of added HCP from brain tissue averaged 94% with a level of 1·0 μg/g of tissue and 83% at 0·125 μg/g. This procedure probably measures only unconjugated HCP, as it is unlikely that the glucuronide would be acetylated.

Radioactivity measurements. To determine the incorporation of precursor compounds into major cellular chemical components, rats were dosed orally with 10 mg HCP/kg from 2–17, 3–18, 16–18 or 2–37 days of age and then injected ip with the paired compounds [^{3}H]mevalonic acid (20 μCi) and [^{32}P]phosphoric acid (100 μCi) or with [^{14}C]leucine (10 μCi) and [^{14}C]adenine (10 μCi). All radioactive compounds were purchased from New England Nuclear Corp., Boston, Mass. 02118. After either 30 or 60 min, the rats were decapitated and quickly drained of blood. The brains and livers were removed, immediately homogenized in ice-water and fractionated according to a scheme based in part on the work of Munro & Fleck (1966) and outlined in Fig. 1, to yield ATP, phospholipid, sterol, DNA, RNA and protein. Portions of each fraction were solubilized with NCS reagent (Amersham/Searle, Arlington Heights, Ill. 60005) and scintillation fluid (0·4% diphenyloxa-

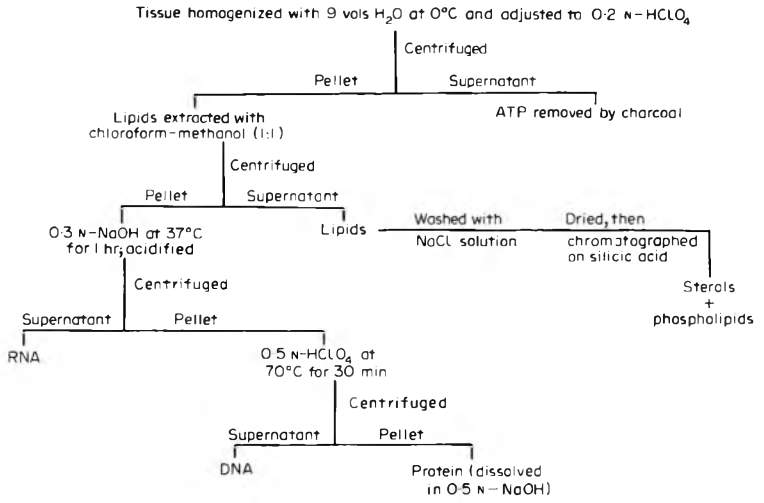

Fig. 1. Outline of isolation procedure for chemical components of brain and liver.

zole in toluene) was added. The samples were precooled overnight in the dark and counted in a Packard Tricarb scintillation counter (Packard Instrument Co., Downers Grove, Ill. 60515). Phosphorus-32 and tritium were counted with approximately 1% spillover into the tritium channel while the carbon-14 was counted in the third channel. No quench corrections were applied, since identical amounts of similarly treated samples were added for each set and counted two or three times for 20 min each. No corrections for counting efficiencies were made.

Chemical determinations. ATP was determined by the method of Crane & Lipmann (1953) utilizing the procedure of Chen, Toribara & Warner (1956) for phosphorus analysis. Organic phosphorus was determined by the same procedure following ashing with $Mg(NO_3)_2$ (Ames & Dublin, 1960). Sterols were determined by the Liebermann-Burchard method according to Moore & Baumann (1952), and protein was estimated by the method of Lowry, Rosebrough, Farr & Randall (1951). RNA was determined by UV absorption at 260 nm (Munro & Fleck, 1966) and DNA by the diphenylamine reaction (Hatcher & Goldstein, 1969). The chromatographic separation of the lipids is described elsewhere (Ulsamer, Smith & Korn, 1969).

RESULTS

The toxic signs and associated changes in brain morphology of rats dosed with 10 mg HCP/kg/day are shown in Table 1. When nursling rats were dosed with 10 mg HCP/kg once between 6 and 8 days of age and were held until 15–16 days of age, no neurological signs of poisoning developed and the brains were normal on microscopic examination. Four-day-old rats given 1–5 doses of HCP (10 mg/kg) also showed no signs of toxicity, and the brain appeared normal on microscopic examination when they were killed at the age of 5–8 days.

Rats dosed from 2–4 days of age throughout the entire suckling period developed neurological signs of poisoning. These pups began to show tremor when they were 9–13 days old. The tremor became progressively worse for 7–10 days, after which, despite continued

Table 1. *Relation between age and appearance of neurological signs and of changes in brain morphology in rats dosed by gavage with 10 mg HCP/kg/day*

Total no. of rats	Age at start of treatment (days)	No. of doses	Signs of toxicity	No. of rats showing signs of toxicity	Chronology of signs (days after birth)		Pathological examination		Degree of brain-myelin vacuolation
					Onset	Finish	No. of rats	Age when killed (days)	
4	6–8	1	None	—	—	—	—	15–16	—
5	4	1–5	None	—	—	—	5	5–8	—
103	2–4	13–22	Tremor	103	9–13	23–30	6	15, 30, 42	Severe at 15 days to mild at 42 days, in cerebrum, cerebellum and brain stem
			Partial paralysis*	14	8–13	15–24			
21	6–8	2–4	Tremor	21	10–13	22–30	14	15–17	Mild to moderate in brain stem; sometimes in cerebellum, and cerebrum
			Partial paralysis*	6	9–13	—			
30	10–13	3	Tremor	0	—	—	3	13	Mild in cerebrum, cerebellum and brain stem†
			Paralysis	4	After 2nd dose				
			Partial paralysis*	16	After 2nd dose				
			Death	30	After 2nd or 3rd dose				
9	15	3	Tremor	3	21	31			
			Paralysis	4	17	21			
			Death	6	17	21			
6	22	22–23	None	—	—	—	6	52	None, except mild in one area of cerebellum in one rat
6	60	23	None	—	—	—	6	90	None, except mild in one area of cerebellum in one rat

*Impairment of function in one or both front limbs.
†These animals also exhibited mild vacuolation of the cerebellar neocortex.

dosing, it gradually subsided, ending when the rats were 23–30 days old. Furthermore the rats showed impaired movement of their forelimbs, which were held in a flexed position. Similarly, rats given only 2–4 doses of mg HCP/kg/day when 6–8 days old showed tremor and impairment of the function of their forelimbs when they reached 10–13 days of age. These signs gradually subsided over the next 5–10 days.

When dosing was begun at 10–13 days of age the rats were much more susceptible to the toxic effects of HCP and all of them died after the third dose, in contrast to the animals mentioned earlier. Impairment of the function of the forelimbs was again observed but was more severe and resembled paralysis. Toxicity was less severe in rats dosed from 15 days of age and the rats were not affected at all when dosing was started at 22 days of age or later. No control animal exhibited any toxic sign or died of causes seen in the HCP-treated rats, except that a small number of experimental and control rats died as a result of dosing mishaps.

Microscopic changes were consistently observed in the white matter of the brains of rats that showed symptoms of poisoning (Figs 2–5). Vacuolation (status spongiosus) was observed throughout the white matter of rats dosed more than once with HCP from 2–4 days of age. These effects were more pronounced in rats killed at 15 days of age than in those killed at later ages. The vacuolation was similar (although with smaller individual vacuoles in the white matter) to that described previously for adult rats (Kimbrough & Gaines, 1971). The vacuoles were lined with strands of eosinophilic fibres and appeared empty. The lesion was more pronounced in the pons and in the medulla oblongata. This difference may have been due in part to the greater amounts of myelin that had been laid down in the brain stem. The grey matter was generally normal throughout the brain except for those rats that died as a result of dosing started at 11–13 days of age. Here vacuolation of the neocortex where it borders on the white matter was also observed. Whether these vacuoles involved the grey matter or occurred in myelin that traversed these areas could not be determined with certainty. Possibly electron-microscopic examination of these areas might clarify this problem.

In the present study not all the brains were weighed, but in two experiments each involving ten rats dosed for 2 days from the age of 13 or 14 days and killed on the third day, the water content of the brain increased by an average of 10·4 and 5·4%, respectively, over the control brains, and the wet weights of the brains of six animals dosed from 2–16 days of age increased by 8·5% over the brain weights of controls.

In addition to producing neurological signs and changes in brain morphology, HCP produced a decrease in the rate of body-weight gain in young rats. The greatest difference occurred after wk 2 of dosing, when the average weight gain was 42% less than the control weight gain. Some of the animals dosed with 10 mg HCP/kg failed to gain any weight during wk 2 and none gained as much weight as any of the control animals. At the end of wk 3, the HCP-dosed animals had gained an average of 18·9% less than the controls for the week, with some animals gaining as much as controls.

Table 2 lists the HCP concentrations in the blood and brain of rats given 25–30 doses of HCP beginning at 50–60 days of age and killed at different times after the final dose (when 80–90 days of age and weighing 300–400 g). Peak blood levels occurred between 3 and 6 hr after the last dose. The concentration of HCP in blood was greater than that in brain at these early times but was the same 24 hr after the dose. Concentrations of HCP in several tissues (blood, brain, liver and fat) were similar 3, 6 and 24 hr after the final dose of 10 mg/kg in 200–300 g rats dosed either once or 34 times previously (Table 3).

Table 2. *HCP concentrations in the blood and brain of rats in relation to the time after the last dose*

Time after last dose (hr)	No. of rats	Tissue concentrations of HCP	
		Blood (μg/ml)	Brain (μg/g wet tissue)
1·5	2	2·2 (1·5–2·9)	0·4 (0·4–0·4)
3·0	7	5·3 (2·6–10·3)	0·8 (0·6–1·0)
4·0	2	3·5 (3·1–3·9)	0·9 (0·7–1·0)
6·0	5	4·3 (1·8–5·0)	1·1 (0·7–1·4)
7·0	2	1·9 (1·8–1·9)	0·3 (0·2–0·3)
24·0	6	0·4 (0·3–0·6)	0·5 (0·2–0·6)

Rats were 80–90 days old (body weight 300–400 g) when killed and had been given 25–30 daily doses of HCP (10 mg/kg/day) by gavage, beginning at 50–60 days of age. Values for tissue HCP levels are means for the numbers of rats shown, with ranges given in parentheses; the values show a significant difference ($P < 0·01$) with time.

The peak levels in the blood again appeared to occur between 3 and 6 hr, although perhaps closer to 3 hr. Brain and fat contained less HCP than blood or liver but tended to release HCP more slowly. The highest concentration of HCP was found in liver.

Concentrations of HCP in the blood and brain of nursling rats dosed at different ages is shown in Table 4. Blood and brain levels of HCP were examined at either 4–6 or 22–24 hr after the last dose. The data show that 13-day-old rats had the highest concentrations

Table 3. *HCP concentrations in rat tissues in relation to the total number of doses (10 mg/kg/day) given by gavage*

No. of doses†	Time after last dose (hr)	HCP concentration in tissues			
		Blood (μg/ml)	Brain (μg/g wet tissue)	Liver (μg/g wet tissue)	Fat (μg/g wet tissue)
1	3	5·2 (4·4–6·0)	0·5 (0·2–0·7)	8·8 (8·1–9·6)	0·7 (0·4–1·0)
34	3	7·7 (4·5–10·3)	0·8* (0·8–0·8)	14·5 (7·7–18·5)	0·8 (0·6–1·0)
1	6	3·3 (3·2–3·4)	0·8 (0·6–1·1)	10·6 (5·9–13·8)	1·1 (0·9–1·2)
34	6	3·9 (1·8–5·0)	1·2* (1·1–1·3)	11·6 (7·5–15·2)	1·9 (0·6–3·8)
1	24	0·4 (0·4–0·4)	0·6 (0·3–0·9)	0·9 (0·4–2·0)	0·4 –
34	24	0·3 (0·3–0·3)	0·5 (0·4–0·5)	2·2 (0·8–3·3)	0·8 (0·6–0·9)

†Rats given one dose weighed 200–300 g and were 87 days old when killed. Rats given 34 doses weighed 200–300 g when killed and were dosed from days 41 to 87.

Values are means for groups of three rats, with ranges given in parentheses, and those marked with an asterisk are significantly higher than the corresponding value for the group given one dose: *$P < 0·05$. For other groups, there was no significant difference ($P > 0·10$) between the corresponding values for one and for 34 doses.

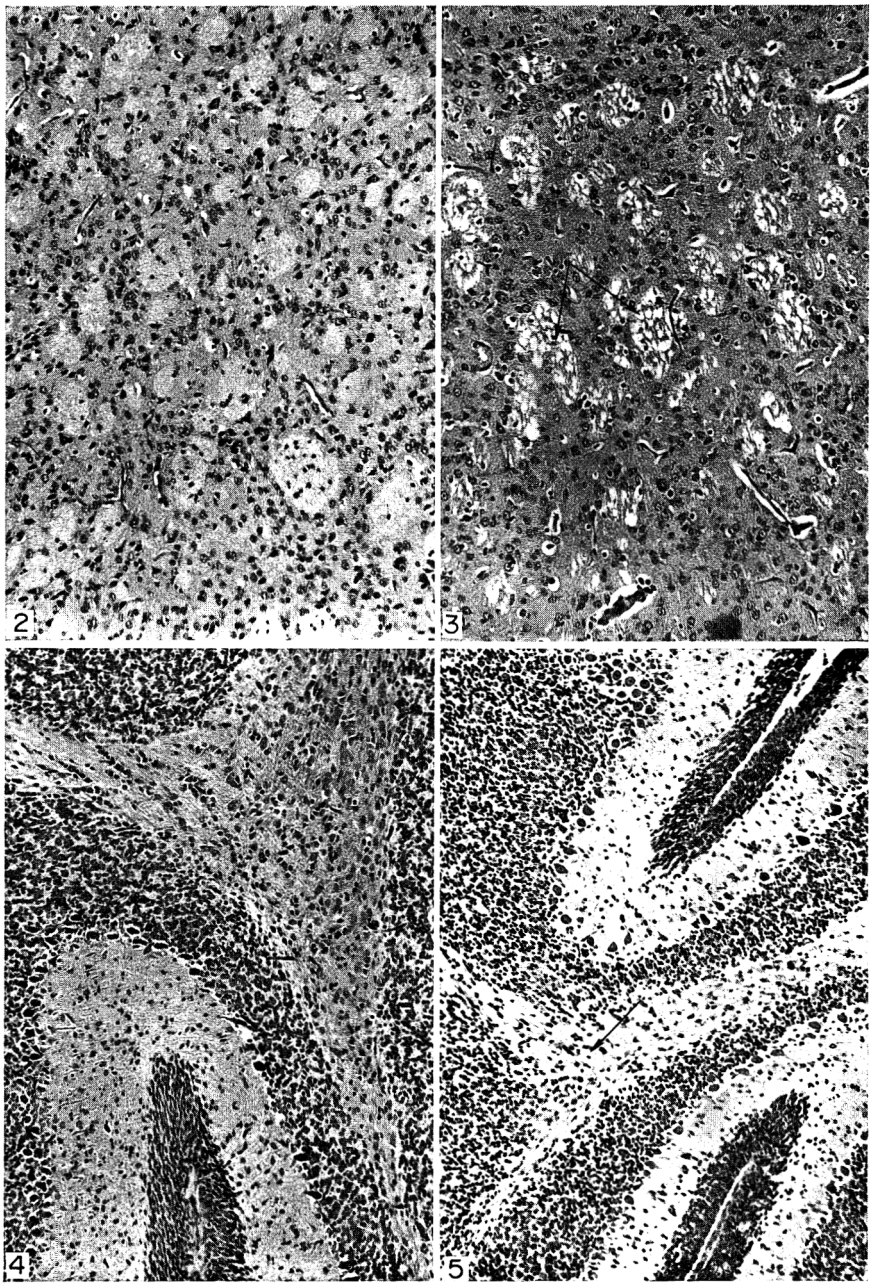

Fig. 2. Section of the deeper white matter of the telencephalon at the level of the anterior commissure of a normal 14-day-old rat. Haematoxylin and eosin × 125.

Fig. 3. Section of the deeper white matter of the telencephalon at the level of the anterior commissure of a 13-day-old rat given HCP in two doses of 10 mg/kg on days 11 and 12 of life. Arrows indicate bundles of vacuolated myelinated nerve fibres. Haematoxylin and eosin × 125.

Fig. 4. Section of cerebellum from untreated rat from which Fig. 2 was derived. Haematoxylin and eosin × 125.

Fig. 5. Section of the cerebellum from the HCP-treated rat from which Fig. 3 was derived. Arrows mark the fine vacuolization of the white matter. Haematoxylin and eosin × 125.

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Table 4. *HCP concentrations in the blood and brain of preweanling rats in relation to age and the number of doses (10 mg/kg/day) given*

No. of doses	Time after last dose (hr)	No. of rats	Age at treatment (days)		Tissue concentrations of HCP	
			Start	Finish	Blood* (μ/ml)	Brain (μg/g wet tissue)
1–2	6	6	4	4–5	8·4 (3·0–12·0)	3·0 (1·1–4·2)
3–5	6	8	4	6–8	9·4 (6·9–13·0)	4·3 (2·5–7·0)
3	4–6	6	11	13	25·4 (23·8–31·6)	10·0 (8·1–13·9)
8–9	4–6	6	3	13	22·6 (16·0–32·0)	8·8 (4·9–13·6)
8–9	22–24	6	3	13–15	4·3† (3·1–6·4)	3·9 (2·4–4·8)
13	24	3	2	20	1·7 (1·3–2·3)	0·7 (0·5–0·9)
18	24	2	3	27	0·1 (0·1–0·1)	0·3 (0·2–0·3)

*The partial correlation between the blood level of HCP and age was positive (r = 0·9999) up to 13 days of age for the 4–6 hr times and negative (r = −0·9753) from 13 to 27 days of age for the 22–24 hr times.
†Mean value for four rats only.
Values are means with ranges given in parentheses.

of HCP in blood and brain at 4–6 hr while the concentrations in 4–8-day-old rats were lower. Comparison of the concentrations at 4–6 hr with the 6-hr data from older rats (Tables 2 & 3) showed lower tissue levels in the older animals.

In an attempt to detect gross metabolic alterations induced by HCP, concentrations of the main chemical components of brain tissue and the uptake of radiolabelled precursor molecules into these compounds were determined. The results of the chemical analysis of whole brain expressed on the basis of wet-tissue weight are given in Table 5. As stated previously, the water content of brains from HCP-treated rats was 5–10% higher than that of control animals. If this is assumed to be true for these animals also, and if biological variation in this period of rapid brain development (12–25 days of age) is considered as well, no consistent changes from control values in total DNA, RNA, ATP, protein, phospholipid or sterol were discernible in HCP-treated rats. Furthermore, assuming that total DNA in HCP-treated rats remained the same as in controls (Friede, 1966; Zamenhof, Bursztyn, Rich & Zamenhof, 1964), still no consistent differences were apparent. The incorporation of radiolabelled precursors into the brain molecules assayed (Table 6) revealed no consistent changes induced by HCP. The specific radioactivities of ATP, phospholipids, sterols, protein and RNA in HCP-treated rats were similar to those in control rats.

DISCUSSION

The appearance of neurotoxic signs induced by HCP coincided with both elevated tissue concentrations of HCP (Table 4) and the period of rapid myelination (12–25 days of age) (McIlwain, 1966). The initiation of the brain lesion at this time appears to result more from a need for myelin than solely from increased HCP concentrations. Thus rats dosed twice (Table 1), starting at 6 to 8 days of age, developed tremors a few days after the final dose of HCP—in the same period (10–11 days of age) as rats dosed continuously from 2 or 3

Table 5. *Effects of 10 mg HCP/kg/day on the chemical components of the brains of developing rats*

Treatment	Age at treatment (days)		No. of rats	Protein* (mg/g wet tissue)	DNA† (mg/g wet tissue)	RNA* (mg/g wet tissue)	ATP† (μmoles/g wet tissue)	Phospholipid† (μmoles/g wet tissue)	Sterol‡ (μmoles/g wet tissue)
	Start	Finish							
Corn oil	2	17	3	62·4 (60·6–66·0)	1·9 (1·5–2·1)	2·6 (2·5–2·7)	4·5 (3·0–5·3)	30·5 (27·7–34·2)	18·0 (14·7–20·6)
HCP	2	17	4	59·7 (49·6–68·7)	1·8 (1·5–2·1)	2·4 (2·1–2·7)	4·3 (3·2–6·5)	27·1 (20·7–41·0)	16·5 (12·7–21·3)
Corn oil	3	18	8	60·0 (59·4–61·1)	1·8 (1·8–1·9)	2·5 (2·4–2·5)	2·8 (2·5–3·1)	31·9 (24·3–42·0)	20·7 (18·5–22·1)
HCP	3	18	8	50·6 (41·6–57·1)	1·7 (1·6–1·8)	2·3 (1·9–2·7)	2·7 (1·9–3·6)	33·7§ (27·6–41·1)	19·8 (18·5–20·9)
	16	18	8	55·5§ (52·5–59·5)	1·7 (1·5–1·8)	2·0 (1·7–2·2)	2·5 (2·2–2·8)	25·1§ (19·6–36·6)	18·6 (17·2–19·9)
Corn oil	2	37	4	65·2 (53·0–74·0)	1·2 (0·9–1·3)	1·9 (1·6–2·0)	2·0 (1·7–2·4)	37·2 (34·1–45·3)	47·8 (43·2–51·2)
HCP	2	37	4	63·3 (55·2–74·3)	1·3 (1·1–1·5)	1·9 (1·8–1·9)	2·0 (1·6–2·9)	30·3 (23·8–35·3)	43·4 (39·0–49·0)

*No significant difference between control and experimental values ($P > 0·10$).
†No significant difference between control and experimental values ($P > 0·25$).
‡Differences of borderline significance ($P > 0·08$) between control and experimental values.
§Only three samples from two rats were used, the other values for these groups and the corresponding control group being means for four samples each from two rats.
Values are means with ranges given in parentheses.

Table 6. *Effects of 10 mg HCP/kg/day on the incorporation of radioactive precursors into the chemical components of the brain*

Treatment	Age at treatment (days)		Isotope exposure time (min)	Specific radioactivity					
	Start	Finish		Protein ($\times 10^4$ cpm/mg)	DNA ($\times 10^4$ cpm/mg)	RNA ($\times 10^4$ cpm/mg)	ATP ($\times 10^3$ cpm/μmole)	Phospholipid ($\times 10^2$ cpm/μmole)	Sterol ($\times 10^2$ cpm/μmole)
Corn oil	2	17	60	1·5*	4·4*	1·6*	3·6	1·7	2·1
HCP	2	17	60	1·6	3·0*	1·8	4·2	1·6	2·5
Corn oil	3	18	30	0·7	6·4	1·0	4·2	1·1	1·8
HCP	3	18	30	0·6	10·4	1·5	4·0	1·0	1·7
	16	18	30	0·6	6·8	1·0*	3·8	1·0	1·6
Corn oil	2	37	60	1·2	7·6	1·8	3·9	0·8*	1·7
HCP	2	37	60	1·5	11·4*	2·5	5·0	1·0	1·6

Pooled results from animals shown in Table 5. Each value is the mean for two animals unless marked with an asterisk, in which case only one animal was used.

days of age. At this time after the last dose, tissue levels of HCP would have been quite low (Tables 2–4). Also, rats sacrificed at 8 days of age showed no evidence of the lesion or of neurological signs, a possible reflection of the low myelin content of the brain at this age. In agreement with this, Seller & Spector (1963) found that rats younger than 10 days of age did not develop brain oedema from water-induced cerebral overhydration, whereas after 10 days of age they did. Similar results were reported following anoxia or ischaemia (Spector, 1961). Especially interesting is the finding that when dosing with HCP (10 mg/kg) was initiated during this critical period of brain development (11–15 days of age) the rats died after two or three doses, whereas those continuously exposed to HCP from the age of 2–4 days did not (Table 1). Blood and brain concentrations of HCP were similar in both groups of animals (Table 4) and no consistent differences were noted in brain concentrations of chemical substituents (Table 5) or in the uptake of radiolabelled precursor molecules (Table 6). The signs of neurotoxicity were different in the two groups, as were the changes in brain morphology (Table 1). The reason for this increase in toxicity when HCP is given at 11–13 days of age in contrast to 2–13 days of age is not known, but could result from inhibition of myelin synthesis at this time or from the synthesis of defective myelin. Also, even though tissue concentrations of HCP were similar in both groups of rats, it is possible that HCP-binding locations could be different. Animals dosed with 10 mg HCP/kg at the end of this period of peak brain development (22 days of age) did not develop the brain lesion, a reflection of lower myelin sensitivity, due perhaps to more complete development, coupled with decreased intestinal absorption and/or more efficient detoxification as well as a better developed blood-brain barrier. R. M. Shuman, R. W. Leech and E. C. Alvord (unpublished data 1974) have noted a similar increase in toxicity extending between 12 and 22 days of ages in rats washed with a cleaner containing 3% HCP. Similarly, Nieminen, Bjondahl & Möttönen (1973) found that oral LD_{50}s were considerably lower in 10–20-day-old rats than in adult rats and were highest in weanling rats.

It has been shown also that HCP is a potent uncoupler of oxidative phosphorylation in liver mitochondria *in vitro* (Caldwell, Nakaue & Buhler, 1972; Cammer & Moore, 1972) as well as in liver mitochondria isolated from rats dosed with HCP (Caldwell *et al.* 1972). The suggestion has been made that this may be the biochemical event that triggers the acute toxic effects of HCP (Caldwell *et al.* 1972; Nakaue, Dost & Buhler, 1973) by producing a rise in body temperature which results in death. Such a rise in body temperature has been noted in adult rats receiving an acute, fatal dose of HCP (Nakaue *et al.* 1973). Alternatively, in longer-term studies, brain oedema itself (or in the case of developing rats, a defect in myelination) may be the immediate cause of death. In our studies no decrease in brain concentrations of ATP or other chemical components was found in HCP-treated rats, as compared with control values (Table 5). In addition, the incorporation of phosphorus-32 into brain ATP and phospholipids was not consistently changed by HCP at either 30 or 60 min after isotope administration, nor was the incorporation of other precursors into product molecules altered (Table 6). Similar results were observed (Stoner & Threlfall, 1958) for rats treated with triethyltin, which produces a brain lesion closely resembling that caused by HCP (Lampert, O'Brien & Garrett, 1973). The fact that no gross biochemical changes were noted in whole brain (Tables 5–6) does not of course rule out such alterations in myelin itself or changes in individual phospholipids, proteins or sterols which might result in abnormal myelin.

Oedema or defective myelination could result from a direct interaction with myelin or myelin precursors. This could be due to binding to a myelin component such as protein,

causing the myelin to split or increase in permeability, whereas in the case of the young rats there may be interaction with a myelin precursor resulting in defective myelination. HCP is known to hydrogen-bond strongly to proteins (Haque & Buhler, 1972) and to increase red-cell fragility (Flores & Buhler, 1971). Also Webster, Ulsamer & O'Connell (1974) showed that after Xenopus tadpoles were immersed in HCP-containing growing solution for up to 7 days, HCP was present in the myelin fraction of brain and that, on the basis of protein content, the amount was twice as great as that of other brain subcellular fractions. Therefore, a variety of factors may be active in the production of the toxic effects of HCP, including both direct actions, such as binding by HCP to protein, and/or indirect actions, such as uncoupling. In addition, the mechanism(s) of action may differ, depending on the age of the animal and the size of the dose administered.

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PERCUTANEOUS PENETRATION OF HEXACHLOROPHENE AS RELATED TO RECEPTOR SOLUTIONS

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Abstract—The *in vitro* penetration of hexachlorophene (HCP) through the stratum corneum was investigated in relation to the receptor solution. Diffusion studies with human stratum corneum showed that 1·0% HCP in propylene glycol achieved a significantly greater ($P < 0{\cdot}025$) mean maximum steady rate of penetration (over 1–24 hr) when normal saline was replaced by 0·2 M-phosphate buffer as the receptor solution. The varying degree of solubility of HCP in the reference solutions studied supports the penetration data. Under the experimental conditions used, HCP was not metabolized as it traversed the stratum corneum.

INTRODUCTION

Hexachlorophene (2,2′-methylenebis-(3,4,6-trichlorophenol); HCP) was synthesized in 1939 (Gump, 1941) and was found to possess a degree of bacteriostatic action against gram-positive bacteria (i.e. Staphylococci). For this reason HCP was incorporated into many cosmetic products and drugs intended mainly for dermal application. Since the integument of the average man has an area of 1·75 sq. m, the potential amount of a penetrating substance entering the body can be large. The barrier to penetration within the integument is the stratum corneum.

Limited data have been reported on the rate of percutaneous absorption of HCP. Carroll, Salak, Howard & Pairent (1967) reported that immersion of the intact rat tail in 3% HCP (pHisoHex®, Winthrop Laboratories, New York) containing added ^{14}C resulted in a penetration rate of 1·7 $\mu g/cm^2/hr$, and the rate was 2·5 times greater through burned skin. Feldmann & Maibach (1970) found that when ^{14}C-labelled HCP (4 mg/cm^2) was applied to the ventral surface of the human forearm, the 5-day absorption rate was 3·10% (124 μg) of the applied dose. Curley, Hawk, Kimbrough, Nathenson & Finburg (1971) reported that no overt toxic symptoms were observed after 50 newborn infants were bathed daily with a 3% HCP solution; at the time the infants were discharged from the hospital their blood levels of HCP ranged from 0·009 to 0·646 $\mu g/ml$. However, the danger of excessive HCP has been well documented following the recent deaths of 28 French babies who had been exposed to talcum powder that contained 6% HCP (Lockhart, 1973). Herter (1959), Lustig (1963) and Pilapil (1966) have further substantiated the toxicity of HCP.

Since the amount and rate of percutaneous absorption of HCP are closely related, the combined effect must be considered in studying its systemic toxicity. This study was initiated to ascertain quantitatively how much HCP penetrates the skin barrier (i.e. the stratum corneum) exposed to different receptor solutions.

EXPERIMENTAL

Diffusion studies. The *in vitro* technique used for the diffusion studies has been described by Marzulli (1962). Aliquots (5 μl) of a 1·0% [methylene-^{14}C]HCP solution (New England Nuclear Corp., Boston, Mass.; specific activity, 1·0 mCi/mmole) in propylene glycol was applied to discs (central contact area, 0·2 cm^2) of human stratum corneum in stainless steel diffusion cells maintained at 37°C. A discontinuous flow of receptor solution (effluent) was used to bathe the undersurface of the stratum corneum to transport the ^{14}C-labelled material out of the diffusion chamber as it traversed the tissue (Marzulli & Brown, 1972). Effluent was collected for chemical analysis at 1, 3, 5, 7, 24, 48 and 72 hr. The effluent was either normal saline, 0·2 M-phosphate buffer (pH 7·4) or 0·2 M-phosphate buffer (pH 7·4) containing 3% bovine albumin (Technicon Instruments Corp., Tarrytown, N.Y.) and brought to ionic strength equivalent to normal saline by dilution either with Ringer's injection USP (Abbott Laboratories, North Chicago, Ill.) or with distilled water. The amount of ^{14}C in each collection was assayed with a Packard Tri-Carb scintillation counter (Packard Instrument Corp., Downers Grove, Ill.). The data were plotted on graph paper with the cumulative amount penetrating as the ordinate and time as the abscissa. The maximum steady rate of penetration was then calculated.

Analyses. A 1·0-ml aliquot of each collection was added to 15 ml of scintillant solution containing per litre: 6 g 2,5-diphenyloxazole, 0·3 g 1,4-bis-2-(4-methyl-5-phenyloxazolyl)-benzene and 100 g naphthalene in 1,4-dioxane. The effluents containing protein were counted in 15 ml Aquasol (New England Nuclear Corp.). The counting efficiencies of both scintillant solutions containing the ^{14}C material were equal and the quench curves were equivalent.

Extraction of [^{14}C]hexachlorophene. After the aliquots for ^{14}C assay were taken from the effluent samples, all remaining volumes were combined, acidified with HCl and extracted with an equal volume of anhydrous ether. The ether phase was retained and evaporated to dryness with a stream of dry nitrogen in a water bath at 60°C, and the residue was taken up in 0·2 ml of benzene. The aqueous phase was assayed for ^{14}C to ascertain the completeness of the extraction procedure and the amount of ^{14}C found was negligible (<0·01%).

Thin-layer chromatography. Glass plates, 5 × 20 cm (Analtech, Inc., Newark, Del.), precoated with silica gel G were used. To each plate 50 μl of the extract was applied 3 cm from the bottom, and the plate was developed in a jar that had been equilibrated overnight with 100 ml heptane–acetic acid (9:1, v/v) until the solvent front was approximately 1 cm from the top of the plate. After removal, the air-dried plate was monitored by a Packard Model 720 radiochromatogram scanner. The detected radioactive area was marked, and the silica gel was removed and placed in the upper end of a Pasteur pipette, which contained fibreglass in the tip end. The radioactive material was eluted from the silica gel into a glass tube with several washings of benzene, and the benzene was evaporated to dryness with a stream of dry nitrogen; 0·5 ml benzene was then added, followed by 0·1 ml pyridine–acetic anhydride (1:1, v/v). The tube was stoppered and the solution was incubated at 60°C

for 15 min. Excess reagent was removed under a gentle stream of dry nitrogen in a water bath at 60°C. This procedure quantitatively converted HCP to the acetyl derivative prior to gas-liquid chromatography.

Gas chromatography. The procedure for the quantitation of HCP has been described by Ulsamer (1972). A column of 3% OV-101 on Gas Chrom Q was used with a ^{63}Ni electron-capture detector.

Solubility of HCP in reference solutions. Saturating amounts of [^{14}C]HCP (New England Nuclear Corp.; specific activity, 1·0 mCi/mmole) were added to 5-ml portions of reference solutions. The closed containers were mixed with the aid of a stirring bar for 24 hr at ambient temperature. The saturated solutions were allowed to settle and then filtered under suction through double Millipore filters (0·22 μ). An aliquot of the filtrate was counted in Aquasol and the concentration of soluble HCP was calculated from specific activity and original volume.

RESULTS

Table 1 gives the mean maximum steady rates of penetration for 1% HCP as it traversed the stratum corneum. By replacing the normal saline with 0·2 M-phosphate buffer (pH 7·4) as the receptor solution used to bathe the undersurface of the skin disc, penetration was enhanced. These data indicate a significant difference ($P < 0{\cdot}025$) in penetration rate. The highest penetration rates were reached with the modified buffer solutions containing protein. Additional evidence for the enhanced rates of penetration of HCP with the different receptor solutions is the solubility of HCP in the respective solutions (Table 2). These data show the following order of solubility: normal saline < 0·2 M-phosphate buffer–water < 0·2 M-phosphate buffer (3% protein)–water < 0·2 M-phosphate buffer (3% protein)–Ringer's solution. This sequence of HCP solubility in the respective buffer solutions corresponds to the relative rates of penetration of HCP obtained in the penetration study, although the values were not quantitatively parallel.

With R_F value as a basis, thin-layer chromatography of the effluent demonstrated that HCP penetrated the stratum corneum without undergoing any metabolism (Fig. 1). This radiochromatogram scan of the effluent gave a single peak which had the same R_F as HCP. In addition, GLC analysis (Fig. 2) showed that the retention time for the single new peak in the experimental stratum corneum extract was the same as that of standard HCP. A stratum corneum blank gas-chromatographed by the methods described showed no peak with a retention time that corresponded to that of standard HCP (Fig. 2); all other

Table 1. *Penetration of 1·0% [^{14}C]HCP in propylene glycol through human stratum corneum in diffusion cells at 37°C*

Receptor solutions	No. of experiments	Mean maximum steady rate (ng/cm^2/hr)	
		1–24 hr	24–72 hr
Normal saline	5	9·6	1·4
0·2 M-Sodium phosphate buffer (pH 7·4)	5	12·1*	2·05
(3% protein†; pH 7·4)	10	17·0*	10·8*
(3% protein†; pH 7·4‡)	10	22·1*	10·8*

†Bovine albumin (globulin 3%, albumin 97%).
‡Ringer's solution used as diluent.
Values marked with an asterisk differ significantly (*$P < 0{\cdot}025$) from the value obtained with normal saline.

Table 2. *Solubility of HCP in reference solutions and penetration rates relative to normal saline*

Solution	Solubility (g/ml)	Values relative to normal saline: Solubility	Values relative to normal saline: Penetration rate (1–24 hr)
Distilled water	$0{\cdot}6.\ 10^{-6}$	—	—
Normal saline	$1{\cdot}0.\ 10^{-6}$	1	1
0·2 M-Sodium phosphate buffer: (pH 7·4)	$21{\cdot}0.\ 10^{-6}$	21	1·3
(3% protein*; pH 7·4)	$1{\cdot}7.\ 10^{-3}$	1700	1·8
(3% protein*; pH 7·4†)	$2{\cdot}1.\ 10^{-3}$	2100	2·3

*Bovine albumin (globulin 3%, albumin 97%).
†Ringer's solution used as diluent.

peaks are natural components of normal (control) stratum corneum. This served to identify HCP under experimental conditions and showed that as HCP traversed the stratum corneum it was apparently not metabolized.

DISCUSSION

The ability of the human skin to metabolize some substances as they traverse the skin is known. Gomez & Hsia (1968) incubated human skin with testosterone and identified several metabolites. Marzulli, Brown & Maibach (1969), employing the *in vitro* method used here, found that the stratum corneum has the ability to metabolize testosterone as it traverses. However, no evidence exists to show that HCP is metabolized in this way.

In man, the stratum corneum is hygroscopic and requires at least 10% moisture to maintain its pliability and softness (Cooper & Lazarus, 1970). The major amino acid constituents of the proteinaceous component(s) of the stratum corneum are aspartyl and glutamyl residues (Crounse, 1963); these residues comprise approximately 22% of the weight

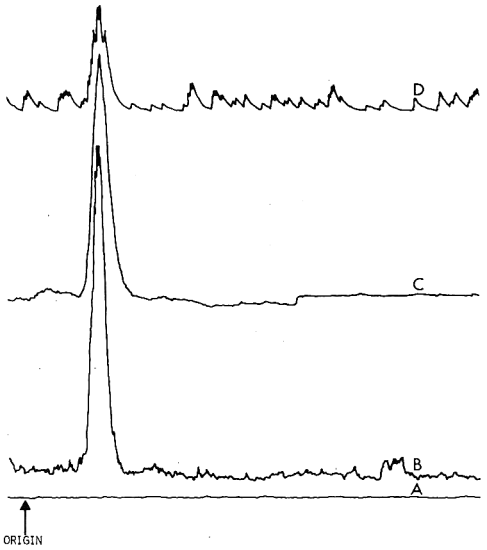

Fig. 1. Scan of thin-layer chromatograms of effluents of stratum corneum: (A) control skin effluent (blank); (B) HCP standard; (C) control skin effluent with standard HCP; (D) effluent from HCP-treated skin.

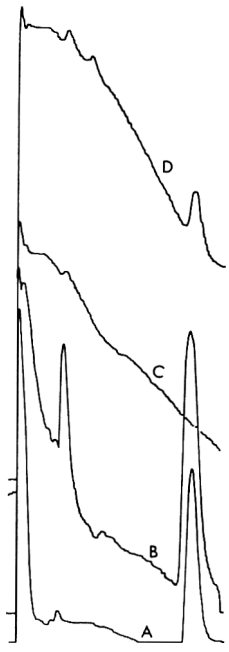

Fig. 2. Gas-liquid chromatograms of effluents of stratum corneum: (A) acetyl derivative of HCP standard; (B) control skin effluent with acetyl derivative of HCP standard; (C) control skin effluent (blank); (D) effluent from HCP-treated skin.

of the keratin of the stratum corneum. HCP is a dihydric phenol and its binding in the skin may occur through hydrogen bonding of the phenols with the peptide linkages (Haque & Buhler, 1972). Pankhurst (1958) suggested that the binding may occur directly by the formation of hydrogen bonding between the phenolic hydroxyl and the peptide nitrogen. Polarity is induced into the aromatic nuclei by the phenolic hydroxyl group, resulting in the development of activated CH groups. The CH group adjacent to the hydroxyl is postulated to be the primary binding site within the molecule. Whatever the mechanism, the bonding is weak and transient, as evidenced by the penetration rate of HCP through the stratum corneum. The increased penetration rates of HCP in the buffer solutions used to bathe the underside of the stratum corneum, as compared with the rate in saline, is partially related to the greater solubility of HCP in the buffers, although the rates did not quantitatively parallel the solubility of HCP in the reference solutions (Table 2). It is apparent that the solubility of HCP in the reference solutions is important, but the rate-limiting factor(s) for penetration is inherent in the stratum corneum. The simulated plasma solutions, 0·2 M-phosphate buffer (3% protein)–water and 0·2 M-phosphate buffer (3% protein)–Ringer's solution, more closely simulate physiological fluids. Therefore, the penetration rates of HCP recorded in this *in vitro* study of human skin should be similar to *in vivo* rates in human subjects using 1·0% HCP.

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SHORT PAPER

IPOMEAMARONE, A TOXIC FURANOTERPENOID IN SWEET POTATOES (*IPOMEA BATATAS*) IN THE UNITED KINGDOM

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Summary—A modified gas-chromatographic procedure for the quantitative measurement of the hepatotoxin, ipomeamarone, in sweet potatoes has been developed and applied in an analytical survey of the ipomeamarone content of retail samples randomly purchased in the UK. Apart from showing the presence of widely varying levels of ipomeamarone in the samples examined, the study provided qualitative evidence for the presence of the lung-oedema factors, 4-ipomeanol and ipomeanine, in several samples.

Introduction

It is now generally recognized that a variety of secondary metabolites, normally absent or present only in low concentrations, may accumulate in plant material subjected to various physiological stresses. These compounds have been called "abnormal" or "stress" metabolites and situations that may lead to their production include microbial infection, physical damage, contact with extraneous chemicals and changes in storage atmosphere or temperature. Antimicrobial compounds produced in response to microbial infections have been called phytoalexins and are regarded as important in the defence mechanisms of plants; the literature of these compounds is inextricably mixed with that of abnormal or stress metabolites and has recently been reviewed (Kuć, 1972) It appears desirable that plant material for human consumption should be examined for the potential production of such compounds, because of their possible pharmacological properties and the possibility that their sensory properties may affect quality and acceptability.

We have mentioned these possible implications in relation to the production of abnormal metabolites in stressed carrots (Coxon, Curtis, Price & Levett, 1973) and we have also reported recently on the isolation of unusual terpenoid derivatives from potatoes (*Solanum tuberosum*) infected with soft rot and potato blight (Coxon, Price, Howard, Osman, Kalan & Zacharius, 1974). A series of publications by Wilson and co-workers (Boyd, Burka, Harris & Wilson, 1974; Boyd & Wilson, 1971; Wilson, 1973; Wilson, Boyd, Harris & Yang, 1971; Wilson, Yang & Boyd, 1970) has shown that, in response to microbial stress, sweet potatoes produce hepatotoxic and lung-oedema factors of a furanoterpenoid type. These factors have had catastrophic effects in cattle consuming mould-damaged sweet potatoes (Peckham, Mitchell, Jones & Doupuik, 1972). The work of Wilson and co-workers followed a long series of papers, principally from Japanese authors (Akazawa, Uritani & Kubota, 1960; Kubota, 1958; Uritani, Uritani & Yamada, 1960), recording the production of compounds of this type in response to a range of different stresses.

The particular report (Boyd & Wilson, 1971) on the presence of these toxic compounds in samples on sale in markets in the USA suggested that it would be of interest to examine material on retail sale in the UK. The market for this vegetable has expanded rapidly with the increase in the immigrant population of the UK—the principal sources are the Canary Islands, the West Indies and West Africa and imports are now about 4000 tons/yr (Bell & Coursey, 1972). It has not been possible to define the original source of the material used in this investigation but it is inevitable that considerable delays between harvest and sale will have occurred and it is likely that there will have been minimal control over storage conditions.

Experimental

Extraction. Sweet potatoes were randomly purchased from retail shops and markets in the Norwich, London, Leeds and Manchester areas during the period from June 1973 to March 1974. Analysis was carried out as soon as possible after purchase but, when necessary, roots were stored for a few days at 15°C prior to analysis. The extraction procedure was a modification of the method of Boyd & Wilson (1971). Whole roots were taken for analysis irrespective of whether they had bruised or otherwise damaged areas, as was often the case. Each root was weighed, shredded and powdered with dry ice by disintegration in a macerator. Duplicate 10 g samples of the thawed material were homogenized in 80 ml acetone. The homogenate was filtered with a sintered Buchner funnel and the residue was washed with 20 ml acetone. The residue was re-extracted twice in the same manner and the combined acetone extracts were evaporated under reduced pressure at 40°C. The residue was partitioned between 100 ml ether and 25 ml water. The aqueous layer was discarded and the ether layer was washed twice more with water, dried over sodium sulphate, filtered and evaporated to dryness.

Analytical procedure. The residue was dissolved in cyclohexane containing methyl palmitate (concentration 2·5 μg/μl) as an internal standard. Aliquots (2 μl) were injected into a Pye 104 gas chromatograph fitted with a glass column (6 ft × 0·25 in. o.d.) containing Diatomite CQ (90–100 mesh) coated with 5% OV 17 (J.J.'s Chromatography Ltd., Kings Lynn, Norfolk). The column was kept at 200°C and the flame-ionization detector at 220°C. The carrier gas was argon at a flow rate of 45 ml/min. Retention times were 11·0 and 7·1 min for methyl palmitate and ipomeamarone, respectively. Quantitative analyses were carried out by relating the area of the ipomeamarone peak (measured by triangulation) to that of the internal standard. The average response ratio (μg ipomeamarone/μg internal standard) was 0·74. Qualitative identification of ipomeanine and 4-ipomeanol was also possible by this procedure.

Results

The results of the analyses are presented in Table 1. Samples from each root were analysed in duplicate, and using the acetone extraction method the duplicate analyses were in very close agreement. In preliminary investigations, chloroform–methanol (95:5, v/v) was used as the extraction solvent (Boyd & Wilson, 1971), but reproducibility was found to be considerably lower. Under the conditions used for the analysis of ipomeamarone, ipomeanine and (+)-4-ipomeanol (Boyd *et al.* 1974) were found to have very similar retention times, of 2·65 and 2·75 min respectively, and were very difficult to distinguish. However, 4-ipomeanol decomposed on injection giving a major dehydration product ($M^{+\cdot}$ 150, by gas chromatography–mass spectrometry) with a retention time of 1·1 min (poorly

Table 1. *Ipomeamarone content of sweet-potato roots*

Sample no.	Condition of root	Ipomeamarone content		Presence* of	
		μg/g fresh weight	mg/root	4-Ipomeanol	Ipomeanine
1	Partially rotten, soft	774	85	+	?
2	Partially rotten, soft	351	39	++	?
3	Partially rotten, soft	871	261	+	?
4	Partially rotten, sprouting	123	11	ND	ND
5	Good	36	4	ND	+
6	Good, sprouting	7	1	+	?
7	Fair, sprouting	61	11	ND	ND
8	Good	ND	ND	ND	+
9	Good	ND	ND	ND	+
10	Internal black speckles	119	17	++	?
11	Good	11	1	++	?
12	Good	1	0·1	+	?
13	Partially rotten, soft	209	18	++	?
14	Good	35	6	ND	ND
15	Good	328	76	ND	+
16	Good	68	13	ND	ND
17	Rotten, soft	883	167	+	?
18	Good	22	7	ND	ND
19	Good	20	4	ND	+
20	Good	1	0·4	ND	ND
21	Good	3	0·7	ND	ND
22	Partially rotten, internal black speckles	844	159	+	?
23	Poor	411	129	ND	+

*ND = not detected; ? = possible presence of ipomeanine obscured due to presence of 4-ipomeanol; + = probably present in small amount; ++ = presence clearly demonstrated.

resolved from the solvent peak) and giving only a very small peak for the original 4-ipomeanol. Despite this problem, 4-ipomeanol was clearly present in four samples and may have been present in small amounts in a further six. These ten samples may also have contained ipomeanine. The latter compound was tentatively identified in a further six samples (see Table) which did not contain 4-ipomeanol, giving no peak at 1·1 min, but which gave a small peak at 2·7 min. In no case was the quantity of ipomeanine or 4-ipomeanol sufficient for their presence to be confirmed by thin-layer chromatography of the crude extract.

Discussion

These results show that sweet potatoes with damaged or microbially infected portions are generally available in the UK and that they may contain significant amounts (100–900 μg/g fresh weight) of the potent hepatotoxin, ipomeamarone. Roots that appeared to be in good condition generally contained much lower levels (<40 μg/g fresh weight), but two samples that were free from any external or internal damage contained 68 and 328 μg/g fresh weight, respectively. Since the Japanese authors have repeatedly emphasized that undamaged sweet potato contains no furanoterpenoids, it is possible either that these two samples had been subjected to some stress which had produced no visually observable effect or that there may be varietal variation which has not so far been noticed. In addition to ipomeamarone there was some qualitative evidence for the presence of small quantities of the lung-oedema factors ipomeanine and 4-ipomeanol in several samples.

Whether the presence of these compounds represents any toxicological hazard is difficult to assess at the present time. These results show that sweet potatoes on sale in the UK contain widely varying levels of ipomeamarone and that these levels are not always predictable from a visual examination of the condition of the roots. Furthermore it has been reported that the compounds are not destroyed by cooking (Wilson *et al.* 1970). The potential damage to health that might be caused by regular ingestion of small quantities of these compounds, probably within a limited segment of the UK population, may require further investigation.

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MONOGRAPHS

Monographs on Fragrance Raw Materials*

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ETHYLENE BRASSYLATE

Synonyms: Ethylene undecane dicarboxylate.
Structure: $H_2\overline{C \cdot OCO \cdot [CH_2]_{11} \cdot OCO \cdot CH_2}$.
Description and physical properties: EOA Spec. no. 188.
Occurrence: Has apparently not been reported to occur in nature.
Preparation: By depolymerization of the polyester of brassylic acid and ethylene glycol (Bedoukian, 1967).
Uses: In public use since the 1940s. Use in fragrances in the USA amounts to less than 2 million lb/yr.

Concentration in final product (%):

	Soap	*Detergent*	*Creams, lotions*	*Perfume*
Usual	0·01	0·001	0·01	0·2
Maximum	0·3	0·015	0·1	3·0

Analytical data: Gas chromatogram, RIFM no. 72–225; infra-red curve, RIFM no. 72–255.

Status

Ethylene brassylate is approved by the FDA for food use (21 CFR 121.1164).

Biological data

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

Sub-acute toxicity. In a 20-day dermal study in New Zealand white rabbits, ethylene brassylate was applied at dosage levels of 30, 70 and 700 mg/kg/day. Dermal irritation was noted. Other than the dermal irritation and enlargement of regional lymph nodes at the 700 mg/kg/day dosage level, no compound related gross pathological lesions or organ weight variations were observed at autopsy or on study of the pathology (Goldenthal, 1974).

Irritation. Ethylene brassylate applied full strength to intact or abraded rabbit skin for 24 hr under occlusion was moderately irritating (Moreno, 1973). Tested at 30% in petrolatum, it produced no irritation after a 48-hr closed-patch test in 25 human subjects (Kligman, 1973).

*The latest of the previous sets of these monographs appeared in *Food and Cosmetics Toxicology* 1974, **12**, 703.

Sensitization. A maximization test (Kligman, 1966) was carried out on 25 volunteers. The material was tested at a concentration of 30% in petrolatum and produced no sensitization reactions (Kligman, 1973).

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Goldenthal, E. I. (1974). Report to RIFM, 7 February.
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Kligman, A. M. (1973). Report to RIFM, 13 July.
Moreno, O. M. (1973) Report to RIFM, 14 May.

ETHYL LAURATE

Synonyms: Ethyl dodecanoate; ethyl dodecylate.
Structure: $CH_3 \cdot [CH_2]_{10} \cdot OCO \cdot CH_2 \cdot CH_3$.
Description and physical properties: *Givaudan Index* (1961).
Occurrence: Reported to be found in nature (*Fenaroli's Handbook of Flavor Ingredients*, 1971).
Preparation: By direct esterification of ethanol with lauric acid under azeotropic conditions (Arctander, 1969).
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	0·03	0·003	0·01	0·2
Maximum	0·3	0·03	0·005	1·2

Status

Ethyl laurate 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 laurate, giving an ADI of 1 mg/kg. The *Food Chemicals Codex* (1972) has a monograph on ethyl laurate and the Joint FAO/WHO Expert Committee on Food Additives (1967) has published a monograph and specifications for ethyl laurate giving a conditional ADI of 1 mg/kg.

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. Ethyl laurate applied full strength to intact or abraded rabbit skin for 24 hr under occlusion was not irritating (Moreno, 1973). Tested at 12% 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 12% in petrolatum and produced no sensitization reactions (Kligman, 1973).

Metabolism. Ethyl laurate is probably hydrolysed to ethyl alcohol and lauric acid, which then undergo normal metabolism (Fassett, 1963).

References

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Kligman, A. M. (1973). Report to RIFM, 12 August.

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

ETHYL METHYLPHENYLGLYCIDATE

Synonyms: Aldehyde C-16; strawberry aldehyde; ethyl α,β-epoxy-β-methylphenylpropionate; ethyl β-methylphenylglycidate.

Structure: $C_6H_5 \cdot C(CH_3)(O) \cdot CH \cdot CO_2 \cdot CH_2 \cdot CH_3$.

Description and physical properties: EOA Spec. no. 109.

Occurrence: Has apparently not been reported to occur in nature.

Preparation: By the reaction of acetophenone and the ethyl ester of monochloracetic acid in the presence of an alkaline condensing agent (Bedoukian, 1967).

Uses: In public use since the 1930s. 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	0·005	0·001	0·03
Maximum	0·05	0·05	0·01	0·05

Status

Ethyl methylphenylglycidate 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 methylphenylglycidate among the artificial flavouring substances not admissible at present including it in the group "Biological Data Indicate Definite Toxicity". The *Food Chemicals Codex* (1972) has a monograph on ethyl methylphenylglycidate and that published by the Joint FAO/WHO Expert Committee on Food Additives (1967) gives a temporary ADI of 0–0·6 mg/kg.

Biological data

Acute toxicity. The acute oral LD_{50} was reported as 5470 mg/kg in the rat and as 4050 mg/kg in the guinea-pig (Jenner, Hagan, Taylor, Cook & Fitzhugh, 1964).

Chronic toxicity. In a feeding study in rats, a dietary level of 10.000 ppm given for 16 wk caused growth retardation, particularly in males, and marked testicular atrophy, while 2500 ppm fed to a similar group for 1 yr produced no effects (Hagan, Hansen, Fitzhugh, Jenner, Jones, Taylor, Long, Nelson & Brouwer, 1967). In a 2-yr feeding study, male and female rats fed a diet containing 5000 ppm ethyl methylphenylglycidate exhibited paralysis of the hindquarters as well as demyelinating degenerative changes in the sciatic nerve (Bär & Griepentrog, 1967). No effect was observed with a dietary level of 1000 ppm, but a subsequent paper (Griepentrog, 1969) reported the finding of effects at all levels when groups of rats were fed diets containing 1000, 3500, 5000 or 6000 ppm ethyl methylphenylglycidate for 2 yr. In these four groups the histological changes of the sciatic nerve were found in 22, 70, 65 and 60% respectively, the effects being marked in 17, 20, 40 and 40% respectively. No histological changes were found in the other organs studied, namely the liver, kidney, spleen and heart.

Irritation. Ethyl methylphenylglycidate tested at a 1% concentration 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 1% in petrolatum and produced no sensitization reactions (Kligman, 1971).

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Kligman, A. M. (1971). Report to RIFM, 24 May.

ETHYL OCTINE CARBONATE

Synonyms: Ethyl-2-nonynoate; ethyl octyne carboxylate.
Structure: $CH_3 \cdot [CH_2]_5 \cdot C{:}C \cdot OCO \cdot CH_2 \cdot CH_3$.
Description and physical properties: A colourless liquid with an odour resembling that of violet leaves (Arctander, 1969).
Occurrence: Has apparently not been reported to occur in nature.
Preparation: From *n*-oct-1-yne via its sodium-derivative, with ethyl chlorocarbonate to the acetylenic ester (Bedoukian, 1967).
Uses: In public use since the 1920s.

Concentration in final product (%)

	Soap	*Detergent*	*Creams, lotions*	*Perfume*
Usual	0·005	0·001	0·001	0·04
Maximum	0·05	0·01	0·01	0·2

Status

Ethyl octine carbonate 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 octine carbonate in the list of admissible artificial flavouring substances, at a level of 1 ppm.

Biological data

Acute toxicity. The acute oral LD_{50} in rats was reported as 2·85 g/kg (1·79–3·91 g/kg) (Moreno, 1973). The acute dermal LD_{50} in rabbits was reported as 5 g/kg (Moreno, 1973).

Irritation. Ethyl octine carbonate applied full strength to intact or abraded rabbit skin for 24 hr under occlusion was not irritating (Moreno, 1973). Tested at 2% 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 2% in petrolatum and produced no sensitization reactions (Kligman, 1973).

References

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Flavoring Extract Manufacturers' Association (1965). Survey of flavoring ingrecient usage levels. No. 2448. *Fd Technol., Champaign* **19**(2), part 2, 155.
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Kligman, A. M. (1973). Report to RIFM, 10 July.
Moreno, O. M. (1973). Report to RIFM. 1 February.

ETHYL PHENYLACETATE

Synonym: Ethyl α-toluate.
Structure: $C_6H_5 \cdot CH_2 \cdot OCO \cdot CH_2 \cdot CH_3$.
Description and physical properties: EOA Spec. no. 40.
Occurrence: Reported to be found in nature (*Fenaroli's Handbook of Flavor Ingredients*, 1971).
Preparation: By ethanolic esterification of the corresponding acid or nitrile.
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	0·05	0·005	0·01	0·2
Maximum	0·2	0·02	0·05	0·8

Analytical data. Gas chromatogram, RIFM no. 72–140; infra-red curve, RIFM no. 72–140.

Status

Ethyl phenylacetate 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 phenylacetate in its list of temporarily admissible artificial flavouring substances. The *Food Chemicals Codex* (1972) has a monograph on ethyl phenylacetate.

Biological data

Acute toxicity. The acute oral LD_{50} value in rats was reported as 3·30 g/kg (2·52–4·08 g/kg) (Moreno, 1973). The acute dermal LD_{50} in rabbits was reported as > 5 g/kg (Moreno, 1973).

Irritation. Ethyl phenylacetate applied full strength on intact or abraded rabbit skin for 24 hr under occlusion was not irritating (Moreno, 1973). When tested full strength for irritation on the forearms of volunteers, it had negative results (Peterson & Hall, 1945), and 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).

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. 169, p. 101. Strasbourg.

Fenaroli's Handbook of Flavor Ingredients (1971). Edited by T. E. Furia & N. Bellanca, p. 392. Chemical Rubber Co., Cleveland, Ohio.

Flavouring Extract Manufacturers' Association (1965). Survey of flavoring ingredient usage levels. No. 2452. *Fd Technol., Champaign* **19**(2), part 2, 155.

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Moreno, O. M. (1973). Report to RIFM, 1 February.
Peterson, H. R. & Hall, A. (1945). Dermal irritating properties of perfume materials. *Drug Cosmet. Ind.* **58**, 113.

ETHYL PHENYLGLYCIDATE

Synonyms: Ethyl 3-phenylglycidate; ethyl α,β-epoxy-α-phenylpropionate.
Structure: $C_6H_5 \cdot CH(O)CH \cdot OCO \cdot CH_2 \cdot CH_3$.
Description and physical properties: EOA Spec. no. 246.
Occurrence: Apparently has not been reported to occur in nature.
Preparation: By the reaction of benzaldehyde and the ethyl ester of monochloracetic acid in the presence of an alkaline condensing agent (Bedoukian, 1967).
Uses: In public use since the 1930s. 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	0·15
Maximum	0·1	0·01	0·05	0·4

Analytical data: Gas chromatogram, RIFM no. 72–141; infra-red curve, RIFM no. 72–141.

Status

Ethyl phenylglycidate 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 phenylglycidate in its list of temporarily admissible artificial flavouring substances. The *Food Chemicals Codex* (1972) has a monograph on ethyl phenylglycidate.

Biological data

Acute toxicity. The acute oral LD_{50} value in rats was reported as 2·3 ml/kg (2·0–2·6 ml/kg) (Shelanski, 1973b). The acute dermal LD_{50} value in rabbits was reported as > 5 g/kg (Shelanski, 1973a).

Irritation. Ethyl phenylglycidate applied full strength to intact or abraded rabbit skin for 24 hr under occlusion was not irritating (Shelanski, 1973a). Tested at 4% 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 4% in petrolatum and produced no sensitization reactions (Kligman, 1973).

References

Bedoukian, P. Z. (1967). *Perfumery and Flavoring Synthetics*. 2nd ed., p. 297. 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 2, no. 102, p. 99. Strasbourg.
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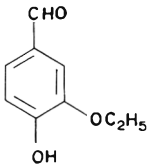
Kligman, A. M. (1973). Report to RIFM, 9 May.
Shelanski, M. V. (1973a). Report to RIFM, 30 January.
Shelanski, M. V. (1973b). Report to RIFM, 20 February.

ETHYL VANILLIN

Synonyms: 3-Ethoxy-4-hydroxybenzaldehyde; protocatechuic aldehyde-3-ethyl ether.
Structure:



Description and physical properties: *Givaudan Index* (1961).
Occurrence: Has apparently not been reported to occur in nature.
Preparation: By ethylation of protocatechualdehyde (Bedoukian, 1967).
Uses: In public use since the 1930s. Use in fragrances in the USA amounts to less than 28,000 lb/yr.

Concentration in final product (%):

	Soap	*Detergent*	*Creams, lotions*	*Perfume*
Usual	0·01	0·003	0·003	0·12
Maximum	0·10	0·025	0·06	0·20

Analytical data: Gas chromatogram, RIFM no. 70–11; infra-red curve, RIFM no. 70–11.

Status

Ethyl vanillin was granted GRAS status by FEMA (1965) and is approved by the FDA for food use (GRAS). The Council of Europe (1970) listed ethyl vanillin, giving an ADI of 10 mg/kg. The *Food Chemicals Codex* (1972) and the *National Formulary* (1970) each has a monograph on ethyl vanillin and the Joint FAO/WHO Expert Committee on Food Additives (1967) has published a monograph and specifications for ethyl vanillin giving an unconditional ADI of 0–10 mg/kg.

Biological data

Acute toxicity. The acute oral LD_{50} value in rats was reported as >2000 mg/kg (Jenner, Hagan, Taylor, Cook & Fitzhugh, 1964) and the acute oral LD in rabbits as 3000 mg/kg (Deichmann & Kitzmuller, 1940). The sc LD in rats was reported as 1800 mg/kg (Deichmann & Kitzmuller, 1940). The ip LD_{50} was reported as 750 mg/kg in mice and as 1140 mg/kg in guinea-pigs, while the iv LD in dogs was reported as 760 mg/kg (Caujolle & Meynier, 1954).

Long-term toxicity. In feeding studies, 20 mg/kg body weight/day fed to rats for 18 wk produced no effects, while rats fed 64 mg/kg/day for 10 wk showed a reduction in growth rate and myocardial, renal, hepatic, lung, spleen and stomach injuries (Deichmann & Kitzmuller, 1940).

Neither 20,000 and 50,000 ppm fed to male rats in the diet for 1 yr nor 5000, 10,000 and 20,000 ppm fed to male and female rats in the diet for 2 yr produced any effects (Hagan, Hansen, Fitzhugh, Jenner, Jones, Taylor, Long, Nelson & Brouwer, 1967).

Irritation. Ethyl vanillin tested at 2% in petrolatum produced a mild irritation after a 48-hr closed-patch test in 25 human subjects (Kligman, 1970).

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, 1970).

References

Bedoukian, P. Z. (1967). *Perfumery and Flavoring Synthetics.* 2nd ed., p. 344. Elsevier Publishing Co., New York.

Caujolle, F. & Meynier, D. (1954). *C.r. hebd. Seanc. Acad. Sci., Paris.* **238,** 2576.

Council of Europe (1970). Natural and Artificial Flavouring Substances. Partial Agreement in the Social and Public Health Field. List A(1). Series 1, no. 109, p. 54. Strasbourg.

Deichmann, W. & Kitzmuller, K. (1940). *J. Am. pharm. Ass.,* **29,** 425.

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Hagan, E. C., Hansen, W. H., Fitzhugh, O. G., Jenner, P. M., Jones, W. I., Taylor, Jean M., Long, Eleanor L., Nelson, A. A. & Brouwer, J. B. (1967). Food flavourings and compounds of related structure. II. Subacute and chronic toxicity. *Fd Cosmet. Toxicol.* **5,** 141.

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.

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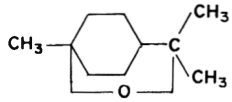
National Formulary (1970). 13th ed. Prepared by the National Formulary Board. p. 303. American Pharmaceutical Association, Washington, D.C.

EUCALYPTOL

Synonyms: 1,8-cineole; 1,8-oxido-*p*-menthane; 1,8-epoxy-*o*-menthane.
Structure:



Description and physical properties: EOA Spec. no. 288.
Occurrence: Eucalyptol forms the main constituent of certain Eucalyptus species. It is found in several hundred oils, including cardamom, ginger root, spike lavender and rosemary (Gildemeister & Hoffman, 1966; Guenther, 1949).
Preparation: By separation from essential oils by freezing or by a combination of distilling and freezing.
Uses: In public use since the early 1900s. Use in fragrances in the USA amounts to less than 5000 lb/yr.

Concentration in final product (%)

	Soap	*Detergent*	*Creams, lotions*	*Perfume*
Usual	0·03	0·005	0·02	0·2
Maximum	0·4	0·04	0·2	1·6

Analytical data: Infra-red curve, RIFM no. 72-143.

Status

Eucalyptol 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 eucalyptol in its list of admissible artificial flavouring substances, at a level of 15 ppm.

Biological data

Acute toxicity. The acute oral LD_{50} value in rats was reported as 2480 mg/kg (Jenner, Hagan, Taylor, Cook & Fitzhugh, 1964). The acute dermal LD_{50} value in rabbits exceeded 5 g/kg (Moreno, 1972).

Irritation. Eucalyptol applied full strength to intact or abraded rabbit skin for 24 hr under occlusion was not irritating (Moreno, 1972). Tested at 16% in petrolatum, it produced no irritant effects 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 16% in petrolatum and produced no sensitization reactions (Kligman, 1972).

Metabolism. Eucalyptol undergoes oxidation *in vivo* with the formation of hydroxycineole, which is excreted as hydroxycineoleglucuronic acid (Williams, 1959).

Additional published data

The administration of eucalyptol to rats (Jori, Bianchetti & Prestini, 1969; Jori, Bianchetti, Prestini & Garattini, 1970) increased the activity of drug-metabolizing enzymes in the liver. Furthermore, animals pretreated with eucalyptol showed a reduced sensitivity to pentobarbitone and lower plasma and brain concentrations of the barbiturate (Jori *et al.*, 1969 & 1970). Eucalyptol also increased the microsomal activity of rat liver after a

single sc dose. Further doses did not enhance the effect. Eucalyptol did not affect the concentration of cytochrome *P*-450 in liver microsomes (Jori, Di Salle & Pescador, 1972).

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EUCALYPTUS OIL

Description and physical properties: *Food Chemicals Codex* (1972). The chief constituent of eucalyptus oil is eucalyptol* (Guenther, 1950).
Occurrence: Found in the leaves of *Eucalyptus globulus* Labill. and other species of Eucalyptus L'Heritier (Fam: Myrtaceae) (Guenther, 1950).
Preparation: By steam distillation of the leaves of *E. globulus* Labill. and other species of Eucalyptus L'Heritier (Gildemeister & Hoffman, 1961; Guenther, 1950).
Uses: In public use since before the 1900s. Use in fragrances in the USA amounts to less than 32,000 lb/yr.

Concentration in final product (%):

	Soap	*Detergent*	*Creams, lotions*	*Perfume*
Usual	0·03	0·005	0·02	0·10
Maximum	0·3	0·04	0·1	1·0

Analytical data: Gas chromatogram, RIFM nos 70–14, 73–19; infra-red curve, RIFM nos 70–14, 73–19.

Status

Eucalyptus oil was granted GRAS status by FEMA (1965) and is approved by the FDA for food use (21 CFR 121.1163). The Council of Europe (1970) included eucalyptus 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. Monographs on eucalyptus oil have been published in the *Food Chemicals Codex* (1972) and in the *National Formulary* (1970).

Biological data

Acute toxicity. The acute oral LD_{50} value of eucalyptol was reported as 2480 mg/kg in the rat (Jenner, Hagan, Taylor, Cook & Fitzhugh, 1964). The acute dermal LD_{50} in rabbits exceeded 5 g/kg (Moreno, 1973).

Irritation. Undiluted eucalyptus oil applied to the backs of hairless mice was not irritating (Urbach & Forbes, 1973). When applied full strength to intact or abraded rabbit skin for 24 hr under occlusion, it was moderately irritating (Moreno, 1973). Eucalyptus oil tested at 10% in petrolatum produced no irritation after a 48-hr closed-patch test in 25 human subjects (Kligman, 1973), and a patch test using the full strength 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 10% in petrolatum and produced no sensitization reactions (Kligman, 1973).

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

Metabolism. 1,8-Cineole (eucalyptol), the chief constituent of eucalyptus oil, apparently undergoes oxidation *in vivo* with the formation of hydroxycineole which is excreted as hydroxycineoleglucuronic acid (Williams, 1959).

*See this issue p. 105.

Additional published data

Eucalyptus 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). It has been shown to be rapidly absorbed through the skin of the mouse (Meyer & Meyer, 1959).

Hypersensitivity from eucalyptus oil has been reported by Goodman & Gilman (1942), Löwenfeld (1932), Schwartz & Peck (1946) and Schwartz, Tulipan & Peck (1947).

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CASSIA OIL

Description and physical properties: *Food Chemicals Codex* (1972). The main constituent of cassia oil is cinnamic aldehyde (Guenther, 1950).
Occurrence: Found in the leaves and twigs of *Cinnamomum cassia* Blume (Fam: Lauraceae) (Guenther, 1950).
Preparation: By distillation of the leaves and twigs of *C. cassia* Blume (Guenther, 1950).
Uses: In public use since the 1800s. Use in fragrances in the USA amounts to about 9000 lb/yr.

Concentration in final product (%):

	Soap	*Detergent*	*Creams, lotions*	*Perfume*
Usual	0·01	0·001	0·005	0·05
Maximum	0·1	0·01	0·03	0·4

Analytical data: Gas chromatogram, RIFM no. 72–92; infra-red curve, RIFM no. 72–92.

Status

Cassia oil was granted GRAS status by FEMA (1965) and is approved by the FDA for food use (GRAS). The Council of Europe (1970) included cassia oil in its 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 cassia oil.

Biological data

Acute toxicity. The acute oral LD_{50} value in rats was reported as 2·8 ml/kg (2·5–3·2 ml/kg) and the acute dermal LD_{50} value in rabbits as 0·32 ml/kg (0·21–0·48 ml/kg) (Shelanski, 1972).

Irritation. Undiluted cassia oil applied to the backs of hairless mice was mildly irritating (Urbach & Forbes, 1972), and applied to intact or abraded rabbit skin for 24 hr under occlusion was severely irritating (Shelanski, 1972). Tested at 4% in petrolatum, cassia oil produced no irritation after a 48-hr closed-patch test in 25 human subjects (Kligman, 1972). A patch test of the full-strength oil on 24 human subjects for 24 hr produced two reactions (Katz, 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 two sensitization reactions (Kligman, 1972).

Phototoxicity. Low-level phototoxic effects were reported for cassia oil, but were not considered significant (Urbach & Forbes, 1972).

Additional published data

Cassia oil is listed as a sensitizer by Schwartz & Peck (1946) and Schwartz, Tulipan & Peck (1947). A girl employed in dipping toothpicks in oil of cassia had a skin reaction affecting the hands, face and abdomen (Prosser-White, 1923; White, 1897). Irritation may occur in hypersensitive individuals (Tulipan, 1938).

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CINNAMON BARK OIL, "CEYLON"

Description and physical properties: EOA Spec. no. 87. The chief constituent of cinnamon bark oil is cinnamic aldehyde (Gildemeister & Hoffman, 1959; Guenther, 1950).
Occurrence: Found in the bark of the shrub *Cinnamomum zeylanicum* Nees (Fam: Lauraceae).
Preparation: By steam distillation of the dried inner bark of the shrub *C. zeylanicum* Nees (Guenther, 1950).
Uses: In public use since the 1860s. Use in fragrances in the USA amounts to less than 4000 lb/yr.

Concentration in final product (%):

	Soap	*Detergent*	*Creams, lotions*	*Perfume*
Usual	—	—	0·003	0·09
Maximum	—	—	0·01	0·8

Analytical data: Gas chromatogram, RIFM no. 72–99; infra-red curve, RIFM no. 72–99.

Status

Cinnamon bark oil, Ceylon, was granted GRAS status by FEMA (1965) and is approved by the FDA for food use (GRAS). The Council of Europe (1970) included cinnamon bark oil, Ceylon, in its 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 cinnamon bark oil, Ceylon.

Biological data

Acute toxicity. The acute oral LD_{50} value in rats was reported as 3·4 ml/kg (2·9–4·0 ml/kg) and the acute dermal LD_{50} value in rabbits as 0·69 ml/kg (0·58–0·82 ml/kg) (Shelanski, 1972).

Irritation. Undiluted cinnamon bark oil, Ceylon, applied to the backs of hairless mice was mildly irritating (Urbach & Forbes, 1972), and applied to intact or abraded rabbit skin for 24 hr under occlusion was severely irritating (Shelanski, 1972). Tested at 8% in petrolatum, the oil produced no irritation after a 48-hr closed-patch test in 25 human subjects (Kligman, 1972; RIFM no. 72–8–99). Retested at 8% in petrolatum, it again produced no irritation (Kligman, 1973; RIFM no. 72–8–248R).

Sensitization. A maximization test (Kligman, 1966) was carried out on 25 volunteers. The material (RIFM no. 72–8–99) was tested at a concentration of 8% in petrolatum and produced 18 sensitization reactions (Kligman, 1972). A further maximization test (Kligman, 1966) carried out on 25 volunteers using the material (RIFM no. 72–8–248R) at a concentration of 8% in petrolatum produced 20 out of 25 sensitization reactions (Kligman, 1973).

Phototoxicity. Low-level phototoxic effects were reported for cinnamon bark oil, Ceylon, but were not considered significant (Urbach & Forbes, 1972).

Metabolism. Cinnamic aldehyde, the chief constituent of cinnamon bark oil, is oxidized to cinnamic acid, which is then degraded to benzoic acid (Williams, 1959).

Additional published data

Three cases of acute contact sensitivity to a dentifrice have been reported. Subsequent testing attributed these reactions to cinnamon oil (Millard, 1973).

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Review Section

BOOK REVIEWS

Recent Advances in Studies on Cardiac Structure and Metabolism. Vol. 2. Cardiomyopathies. Edited by E. Bajusz and G. Rona. Medical and Technical Publishing Co. Ltd., Lancaster, 1973. pp. xiii + 842. $39.50.

It is common knowledge that heart disease arises as a consequence of arterial blockage, but the heart muscle is susceptible to a variety of other disorders which are of uncertain aetiology and which are grouped together as cardiomyopathies. The volume under review provides a useful collection of articles on this somewhat ill-defined group of cardiac disorders.

Considerable attention is given to the spontaneous cardiomyopathies that afflict man. B. McKinney and T. G. Ashworth describe fully a type of heart disease that is prevalent in tropical Africa and is called endomyocardial fibrosis. The pathology and clinical history of this condition is contrasted by these and other authors with the so-called cardiomegaly of unknown origin found in South Africa and with a variant of the same type of disease found in Rhodesia. J. F. Goodwin gives a comprehensive account of the cardiomyopathies that occur in the United Kingdom. The so-called 'congestive' type is associated with dilatation of the heart and poor function, and is usually the end result of untreated hypertension. A less common disorder is the 'hypertrophic' type, in which gross enlargement of the heart muscle may, and often does, result in obstruction of the blood flow.

In addition, the heart becomes involved in a variety of generalized disease processes, such as amyloidosis and sarcoidosis. The incidence of heart disease attributable to excessive alcohol consumption—so-called alcoholic cardiomyopathy—is very difficult to determine, but two American contributors, G. E. Burch and T. D. Giles are of the opinion that this type of heart disease is not only common but is "a serious, important, and frequently overlooked clinical problem", at least in the USA.

Apart from these cardiomyopathies, in which the aetiological agent is generally obscure, there is a group of myocardial diseases which are attributable to specific viruses. These are discussed in a series of chapters dealing with clinical studies, epidemiology and pathology, as well as with virus identification. The reader is left with the impression that of all the cardiomyopathies the ones due to viruses are the best documented—a credit to virologist and pathologist alike.

Another section of the book pays considerable attention to subcellular changes in some of the cardiomyopathies, but despite the diversity of the findings at biochemical and ultrastructural levels, there is little here to help the diagnostician to identify the cause of the disease.

Three sections are devoted entirely to cardiomyopathies in experimental animals and some of the descriptions of spontaneous types include useful comparisons with similar

forms of the disease in man. Another section is of considerable interest to the food toxicologist, since it contains accounts of the heart-muscle lesions induced by cobalt and by rapeseed oil. The cobalt-induced heart disease in heavy beer-drinkers is fully described by G. Rona and C. I. Chappel and is compared with the disease induced experimentally in laboratory animals. The cardiotoxic effects of rapeseed oil are adequately described by H. A. Heggtveit, E. A. Nera and J. L. Beare-Rogers, but unfortunately little attention is paid to the implications of these findings with regard to man.

This collection of papers will be of value principally to clinicians and experimentalists engaged in investigating the causes of human cardiomyopathy. However, the toxicologist, and particularly the pathologist engaged in the diagnosis of morbid processes in animals in connexion with toxicity studies, could with profit consult the text for clues to assist in the diagnosis of obscure heart lesions.

Sub-clinical Lead Poisoning. By H. A. Waldron and D. Stöien. Academic Press, London, 1974. pp. x + 224. £5.50.

For those who consider that every human extravagance is rewarded by a comparable degree of discomfort, there is no more appropriate example than that of man's confrontation with the lead in his environment. In Roman times, lead-lined vessels and pipes were used to store wine and supply water, while in our own age the largely industrial use of lead and the use of alkyllead compounds as motor fuel additives has resulted in the release of considerable amounts into the general environment. It is ironic that, in discovering more sophisticated applications for this ubiquitous heavy metal, man has altered the picture of lead poisoning from that of an overt clinical syndrome to a more insidious pattern of long-term effects. It is this "new dimension" in the toxicological spectrum of lead that has formed the basis for a factual and clearly presented text aimed at evaluating information from many sources relevant to the problem of subclinical lead poisoning. The framework for this discussion takes the form of more than a thousand relevant references, embracing a range of subject-matter from historic background to prognostic criteria.

To set the scene, the authors have wisely included an account of the environmental lead burden and of the ways in which the animal body metabolizes inorganic and organic forms of lead. In a chapter describing the pathological effects of lead, we are informed that histological anomalies found in lead neuropathy have been studied in detail for almost a century, yet the agent directly responsible for these lesions remains obscure. The authors put forward an interesting theory suggesting that the two most common manifestations of neuropathy—segmental demyclination and capsular degeneration—may be linked through a common metabolic lesion affecting the supporting cells of the dorsal root ganglion and the Schwann cells of the peripheral nervous system, especially since these two cell types have a common embryonic origin.

In considering the possible implications of effects associated with persistent exposure to lead, the authors have collated data from three major sources. Primarily, attention is focused on evidence for associations between a high lead burden in childhood and impaired mental development in the young. The seventies have seen a step forward in this area, since several studies have centred on the possible psychological as well as the neurological sequelae of lead exposure. Animal experiments have contributed little to our understanding of lead-induced behavioural effects—a possible reflection of the inadequacy of

current psychological testing procedures, which leave much to be desired. Finally, the significance of environmental lead is considered in the context of epidemiological evidence that lead is associated with ailments such as cardiovascular disease and multiple sclerosis. The authors have struck the correct balance here by emphasizing that the almost inextricable relationship between social, dietary and other environmental factors in the epidemiology of these diseases makes it extremely difficult to isolate a single toxic principle from the array of possible causative agents.

Two final chapters, concerning the diagnosis and the prophylaxis and treatment of lead poisoning are of interest to the toxicologist in so far as they are dependent on reference values which distinguish the pathological from the "non-pathological" case. The assertion that individual laboratories should establish their own values by recourse to a suitable reference population certainly seems to offer the wisest course at present.

The existence of a lower safety margin for environmental lead than for any other recognized chemical pollutant should be sufficient stimulus for investigation of the more surreptitious implications of long-term exposure in man. Plainly, the message of this book lies not in any pessimistic assertion that 'our daily lead' is waging a silent war on human health, but that far too little is known about the extent to which environmental lead actually produces harmful physiological or psychological effects.

Environment and Birth Defects. By J. G. Wilson. Academic Press. London, 1973. pp. xiv + 305. £8.95.

Man's tendency to accept abnormalities among his offspring as purely chance occurrences has always been accompanied by a sense of morbid curiosity which, until recent times, found its expression in fantasy and superstition. Only during this century has this curiosity been subjected to the discipline of scientific enquiry, the more recent stages of which are carefully recorded in a new text written by an expert in the field of experimental teratology.

Opening with a discussion of possible risks to the unborn organism from environmental factors, the author provides a strong counter-attack to the attitude, prevalent in the early part of this century, that all abnormal development is genetically determined. The tragedy of thalidomide made it eminently clear that "if a single drug could cripple some 8000 children in a span of two years", there was more than sufficient reason to probe the possible effects of other environmental agents on development. Unfortunately, the situation is often complicated by a multiplicity of interacting phenomena, as evidenced by the fact that some extrinsic factors have been shown to be synergistic in their abilities to induce malformations. Nevertheless, some reassurance may be derived from the author's assertion that postnatal functional disorders represent the only area of compound-specific embryotoxicity for which the existence of a no-effect level can reasonably be doubted at present.

It is of interest that only a small proportion of human developmental abnormalities of known cause can be attributed to environmental chemicals, but on the other hand it must be remembered that for some 70% of developmental defects the causative factor has not been identified. One possible cause that has received much attention in recent times is the perinatal nutritional deficiency common among individuals whose standard of living is low. Specific nutritional deficits are known to interfere with structural and functional development in animals, but no definite conclusions can be drawn at present with regard to a similar situation in man.

Susceptibility to environmental influences is greatest during organogenesis, but the author takes care to stress that the concept of a "critical period" does not preclude the occurrence of malformations at other times during development. The difficulty encountered in attempts to extrapolate the timing of particular events from animals to man is made clear by a useful table in which the most notable species difference in the timing of various stages of intrauterine development in a dozen or so mammals is a surprising variability in the time of implantation.

One of the more valuable messages of this book lies in its pinpointing of the many areas in which teratological experiments can produce misleading results. There is a growing volume of evidence to indicate that long-continued dosage of animals can give results different from those obtained by acute treatment of 'critical' periods in organogenesis, and the possibility that potential teratogenicity may be masked by multiple dosing has been demonstrated. To overcome these problems and enhance the predictive value of teratological testing, the author advocates the integration of long- and short-term treatment groups in the experimental regime.

If there is one lesson that has been learnt over the last decade, it is that experience derived from the testing of one environmental chemical does not always represent a reliable basis for testing new agents. This conviction that there can be no single, all-purpose teratological study leads the author to conclude that if better tests are to be devised, they will involve the use of different levels of evaluation in different types of test animal.

Overall, the merit of this book lies in its careful exposition of the problems inherent in the current situation and the possible lines along which solutions may be sought, rather than in any offer of simple remedies. All readers will appreciate the fluency and clarity with which the author has presented his views.

Liver and Pregnancy. By N. A. M. Bergstein. Excerpta Medica, Amsterdam, 1973. pp. 222. Dfl. 60.00 (*c.* $24).

This book is concerned with the clinical, clinico-biochemical, histological and ultrastructural changes that may occur in the female liver in the course of normal and pathological pregnancies. Chapter 1 deals with the origin, metabolism, conjugation and clearance of bilirubin as it affects the mother and the foetus or newborn. The author pays particular attention to the production and fate of haemoglobin and the perturbation of foetal and maternal bilirubin levels resulting from increased formation or destruction of red blood cells. With something of a jolt, he then turns his attention to the pruritis or itching that frequently accompanies jaundice as a symptom of toxaemia of pregnancy.

The second chapter, on liver function in healthy women, is subdivided into eight sections, six of which indicate the levels of bilirubin, proteins, cholesterol, alkaline phosphatase, transaminases and lactic acid dehydrogenases to be expected in the serum of pregnant and non-pregnant women. The other two sections review the bromsulphthalein tolerance test and the thymol turbidity test (though the author does not state the basis of the latter test or what it measures). Each section takes the form of a literature survey followed by the author's personal findings.

An extensive third chapter is in two parts. The first, entitled "Pregnancy-induced affections of the liver", leaves one in some doubt as to whether the "affections" of the liver are causally related to pregnancy or not, while the second part, "Liver diseases not induced

by pregnancy", appears to investigate the effect of liver pathology on pregnancy rather than *vice versa*. Hepatitis, cirrhosis and gall-stones are among the syndromes covered in some detail before the chapter moves into an interesting, though perhaps irrelevant, list of liver conditions brought about by drugs and tumours.

Chapter 4 deals with the information obtained by liver biopsy and is necessarily short —firstly because needle biopsy has been available only for some 25 years, secondly because of the relatively random character of the specimens obtained, and thirdly because of ethical considerations, since liver biopsy of sick or healthy persons, pregnant or not, is always attended by some risk. In an attempt to classify a rather mixed population of biopsy findings from a score of sources, the author has constructed four tables, arranging patients on the basis of whether pruritis and/or jaundice occurred in the first or subsequent pregnancies. It may perhaps be naïve to point out that the strongest correlation seems to be between the presence or absence of cholestasis and the time of biopsy after parturition or abortion, biopsies performed more than 10 days after parturition rarely showing the presence of jaundice.

A short review of the scanty literature on the electron microscopy of some liver biopsies concludes the chapter. The principal changes in the hepatic ultrastructure of jaundiced pregnant women are said to be enlarged mitochondria with crystalloid inclusions and the presence of abnormal bile canaliculi. It is a pity that there is no mention of the effects of medication on liver ultrastructure, for it is well known that experimental cholestasis induced, for example, by α-naphthyl isothiocyanate (ANIT) or bile-duct ligation, leads to altered bile canaliculi. Similarly it is not uncommon for giant, crystalloid-filled mitochondria to be observed in the livers of patients on prolonged or simultaneous medication with a variety of drugs.

Dr. Bergstein uses chapters 5, 6 and 7 to expound his personal experiences of some 68 pregnant patients, mostly with hyperbilirubinaemia and/or pruritis. They are classified in the same order as were the literature reviews in earlier chapters. The effects of eclamptic toxaemia, diuretics, drug-induced pyelitis, deficiency anaemias, surgical intervention, hepatitis and drugs are explored at length, and therapy is described for the relief of itching.

The use of BSP clearance patterns (a quantitative measure of liver function) for assessing and classifying the degree of hyperbilirubinaemia and pruritis occupies chapter 6, which is supplemented in an appendix by a detailed mathematical treatment of model systems. An account of the author's own histological and ultrastructural examinations of biopsy material forms the final chapter of the book. Case histories of all 68 patients are followed by an extensive bibliography and 19 pages of illustrations, mainly light and electron micrographs and graphs relating liver-function parameters. A full and clear index is provided.

The production is clear and neat, with adequate reproduction of illustrations. Being for the most part clinical or clinico-chemical, this book will probably find a place on the shelves of hospitals rather than of research establishments. Very little of the material included is based on animal experiments, and it is a pity that no histochemical studies were reported. Curiously, pp. 1–4 are marred by typographical and arithmetical errors—what, for example, is "hepatic liver flow" (p. 1)?

Overall, this must be regarded as a valuable source-book for any obstetric clinician who sees jaundice in his patient and must quickly decide on a course of investigation and treatment.

Obesity and its Management. By D. Craddock. 2nd Ed. Churchill Livingstone, Edinburgh, 1973. pp. x + 205. £2.50.

"Let me have men about me that are fat; . . .
Yond' Cassius has a lean and hungry look;
He thinks too much: such men are dangerous."

If the bard is to be believed, obesity should promote harmony and thus confer blessings on society. It appears, however, that obesity confers little blessing on the individual. Not only is he handicapped in having more weight to carry around but his life expectancy is below average.

Many are the recommendations for keeping the weight down, some favouring exercise and most favouring a restricted diet. There is considerable disagreement, however, as to what should be restricted and what should be allowed. Fortunately, this book is not just one more list of 'do's and dont's'. Instead it gives a balanced and reasoned account of the dietary constituents that should be avoided. Furthermore, the author considers obesity as more than one single problem and attempts to distinguish between its various causes in accordance with present-day medical and biochemical knowledge. He then considers the proclivity of some obese people to degenerative disorders and goes on to suggest typical dietary regimens that may be of help.

Although addressed primarily to the medical profession, and to general practitioners in particular, the book is written in a style which will be comprehensible to the lay reader. Many such readers may be surprised to find that, according to the author's strict definition of obesity, they ought to look carefully at their diet!

Biological Nomenclature. By C. Jeffrey. Edward Arnold (Publishers) Ltd., London, 1973. pp. ix + 69. £2.00.

While most biologists and many workers in other sciences must recognize the value of having standardized systems of classification and nomenclature for the enormous range of living organisms, it is probable that relatively few have any particular enthusiasm for the actual mechanics of this process of grouping and definition. One feels, however, that whether it is large or small, the band of enthusiasts includes the author of this little book. His obvious interest in the principles and practice of 'systematics' has enabled him to explain with clarity and relative simplicity the International Codes of Nomenclature and the basis on which they have been formulated.

The introduction to the main text puts the novice on the right path by making a clear distinction between 'classification' and 'nomenclature', the two major branches of systematics, and by outlining the various categories of the taxonomic hierarchy. Subsequent chapters introduce the International Codes used in Botany, Zoology and Bacteriology, describing the forms of names used and the ways in which the three codes differ. Difficulties are inevitable in the operation of a system that depends for its success on stability but is subject to continuing change as knowledge of the organisms concerned increases, and some indication is given of the ways of coping with this problem and with other causes of confusion and ambiguity. The basic rules and principles governing the naming of any organism are described, together with the forms of authority citation often encountered in systematic literature and the variety of other qualifying phrases used. Finally the author turns his attention to certain special groups of organisms which are not fully covered by

the three established codes and for which special provisions have had to be made. These include imperfect fungi, cultivated plants, hybrids—which are recognized in the Botanical Code but for which no provision is made in the Zoological and Bacteriological Codes—domesticated animals and viruses.

A few useful notes supplementing the main text are followed by a bibliography listing official publications on nomenclature and their associated journals as well as the other sources of reference used by the author. A somewhat unusual feature, but one which seems helpful, is the joint compilation of a glossary and index. It is stressed that this book is only a practical guide and introduction to the International Codes of Nomenclature. As such, it will be of value to many who are interested in living organisms either in a professional or amateur capacity.

Pharmacological and Chemical Synonyms. A Collection of Names of Drugs and Other Compounds Drawn from the Medical Literature of the World. By E. E. J. Marler. Excerpta Medica. Amsterdam, 1973. pp. vii + 456. Dfl. 92.00.

Little can be said about this book except that alphabetical lists occupying some 450 pages of double columns offer the user a fair chance of identifying trade-named products or detecting alternative nomenclature for drugs and other compounds of pharmacological and biochemical importance. Within that framework, the scope is wide, international coverage being provided as far as possible.

For each substance covered, there is a single main entry, which lists all the non-proprietary synonyms and trade-names known to the compiler. Each of these, in turn, is listed separately with a cross-reference to the main entry (which is always a non-proprietary name). The foreword provides a clear explanation of the mode of compilation, the scope and limitations of the "dictionary"—such as the principles governing the handling of multiple formulations, vitamins and other biological products—and the procedures adopted in connexion with trade-names. The use of American spelling throughout will not please everybody but provides a welcome consistency in presentation.

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Information Section

ARTICLES OF GENERAL INTEREST

VINYL CHLORIDE: A REPORT OF A EUROPEAN ASSESSMENT

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During 1973, increasing concern was expressed about the possible carcinogenic and other effects of vinyl chloride. The practical implications include the health of industrial operatives exposed to vinyl chloride during the manufacture and processing of PVC. The presence of vinyl chloride in PVC has been unequivocally demonstrated, as has its ability to migrate into food and drink packaged in PVC containers. The migration of vinyl chloride into the atmosphere from PVC is also a strongly presumed, though less documented, probability.

On 26 March 1974 a group of European toxicologists discussed the available toxicological and migration data on vinyl chloride, with special reference to its carcinogenic potential. The meeting was held at The National Institute of Public Health, Bilthoven, The Netherlands. The participants were Dr. Böhme (Federal Republic of Germany), Dr. Carstensen (Denmark), Dr. Crampton (UK), Professor Gatti (Italy), Dr. Kroes and Dr. van Logten (The Netherlands), Professor Schlatter (Switzerland) and Professor Truhaut (France), with Dr. van Esch acting as Chairman.

The essential points of the toxicity and carcinogenicity of vinyl chloride known so far may be summarized as follows:

(1) Inhalation studies with animals show positive carcinogenic effects. In 1971, Viola *et al.* (*Cancer Res.* 1971, **31**, 516) reported tumours of the skin, lungs and bones in rats exposed to a high vinyl chloride concentration (30,000 ppm) in the air of 4 hours daily on 5 days/week for 12 months. The skin tumours arose in the para-auricular region. Reports of further studies carried out in Bologna, Italy (*Chemical and Engineering News*, 1974, 25 February, p. 16) confirm the carcinogenic response and some results are shown in Table 1.

The results shown in Table 1 were obtained in Sprague–Dawley rats exposed to the stated concentrations of vinyl chloride in the inspired air for 4 hours daily on 5 days/week over a period of 12 months. In addition to the angiosarcomas indicated

Table 1. *Carcinogenic response in rats exposed to vinyl chloride in the inspired air*

Exposure level (ppm)	Rats with liver angiosarcoma	No. of rats exposed
10,000	6	69
6000	21	72
2500	9	74
500	7	67
250	2	67
50	0	64

in Table 1, some rats also developed zymbal-gland tumours and nephroblastomas. In concurrent studies, exposure of rats to vinyl acetate at levels up to 2500 ppm was not followed by the appearance of any tumours.

(2) There are strong indications that vinyl chloride exposure results in liver tumours in man. A number of cases of angiosarcoma of the liver have been reported in the United States in operatives exposed to vinyl chloride. This tumour is very rare in man. It is possible that the initial cases recorded will be followed by the appearance of others, and comment on this possibility has already been made by the Center for Disease Control, US Department of Health, Education, and W[illegible]re.

In addition to the possible carcinogenic response in man, vinyl chloride exposure has been associated with the clinical syndrome, acroosteolysis. Full descriptions of the signs and symptoms and clinical findings of this condition have been published (Dinman *et al. Archs envir. Hlth* 1971, **22,** 61; Harris & Adams, *Br. med. J.* 1967, **3,** 712; Jühe *et al. Dt. med. Wschr.* 1973, **97,** 2034; Lange *et al. Int. Archs Arbeitsmed.* 1974, **32,** 1).

(3) Other effects in man have also been recorded (Kramer & Mutchler, *Am. ind. Hyg. Ass. J.* 1972, **33,** 19). In individuals occupationally exposed to concentrations in the air varying from 10 to 300 ppm over periods up to 25 years, indications of liver dysfunction have been observed. This particular effect has not been confirmed in animals, in that inhalation of vinyl chloride by dogs, rabbits and guinea-pigs over a period of 4–6 months has not produced any signs of liver disturbance (Torkelson *et al. ibid* 1961, **22,** 354).

The relationship between the chemical structure of vinyl chloride and its toxicological effects was discussed. A large number of compounds contain double bonds which may lead to free-radical formation. These include styrene, acrylic acid, acrylonitrile, acrylate, vinylidine chloride, vinyl acetate and safrole, a substituted allyl benzene known to have carcinogenic properties in experimental animals. The absence of a carcinogenic response to vinyl acetate indicates that other factors affect its biological activity. Therefore, the reactivity of vinyl chloride with other compounds in the environment and in food and its metabolic route in the body are of considerable importance. Little information on these points is available; for instance, no data exist to indicate the degree, if any, to which vinyl chloride may act as an alkylating agent.

The factors that affect the migration of vinyl chloride from PVC were discussed. In January 1973 the Food and Drug Administration began to receive reports of possible stability problems with PVC bottles used for distilled spirits. The level of vinyl chloride migrating varied, with some samples in a high range of 10–20 ppm. As there were no prior indications that contact between PVC and food would result in migration of monomer, the figure of 10–20 ppm was astonishingly high.

Some data were available for migration into non-alcoholic beverages, food and other materials. It is not possible to include all the basic data, but the following will indicate the order of magnitude of migration. From PVC in which vinyl chloride was present at a level of 30 ppm, migration into water, soft drinks and blood (after 40 days) produced concentrations of 0·02–0·05, 0·0002 and 0·014–0·08 ppm respectively.

The extent of migration depends on the initial content of vinyl chloride in PVC, and this can vary greatly. Thus, in PVC bottles, the content of vinyl chloride may be as low as 5 ppm and as high as 400 ppm (and up to 800 ppm in PVC film). Migration also depends

upon time of storage, as illustrated by a sample of beer which contained 2 ppm vinyl chloride after 6 years in PVC bottles containing 70 ppm.

Other factors that might influence migration are the type of industrial process used, the effects of additives to PVC formulations and conditions of usage. It is known that information on these and other aspects has been generated by industry, and the need for a round-table discussion with industry was agreed by all participants.

On the basis of available migration data, an estimate was made that less than 100 μg vinyl chloride is the likely oral daily intake by man (in Europe). However, further data are needed to quantitate this, and consideration should also be given to compounds formed by the interaction of vinyl chloride with the chemical constituents of food and drink, on which no data were available.

The following conclusions were reached and agreed unanimously:

(1) Industrial exposure to vinyl chloride is the most important problem. Full epidemiological data, including detailed clinical records of operatives with tumours or other alleged effects, should be reviewed. The no-effect level of 50 ppm in inspired air indicated by Professor C. Maltoni is too high to adopt as a Threshold Limit Value*. Present levels of industrial exposure should be reduced as far as possible. Environmental considerations should include the atmospheric effluent from industrial plants. Of particular importance is the elimination of hazard to the operatives from exposure to high levels of vinyl chloride during the cleaning of polymerization vessels.

(2) Methods for reducing the residual vinyl chloride in plastics products to very low levels should be explored urgently. This is of particular importance where PVC is used for food and drink containers and wrappings.

(3) At the present time there is no need to recommend that PVC should be banned as a food-wrapping material, provided active steps are taken as indicated above. The basis of this conclusion is that relatively high concentrations of vinyl chloride (by inhalation) are needed to produce a carcinogenic effect. In comparison, the exposure of man from food intake is probably much lower. The further quantitation of this factor will be possible when oral studies on vinyl chloride have been completed. Furthermore, it was noted that even less toxicological data were available on possible alternative plastics for food wrappings. It was thought desirable and possible that PVC used for food and drink packaging should contain less than 20 ppm vinyl chloride monomer. In effect this represents the establishment of a food grade of PVC, to ensure very low levels of contamination.

(4) The current situation is that the problem of vinyl chloride has been broadly defined. Its final solution depends upon the evaluation of further data, such as:

(a) Epidemiological studies in man concerning vinyl chloride-linked diseases.

(b) Levels of industrial and environmental exposure.

(c) Effects of oral dosing of vinyl chloride in animals.

(d) Investigations on whether the induction of liver tumours by vinyl chloride is preceded by liver dysfunction and cirrhosis, as can be found with selenium, or whether it behaves like nitrosamines, with which no cirrhosis of the liver is observed.

(e) Metabolism of vinyl chloride, including alkylation studies.

* After the meeting it was learned through personal communication that 50 ppm vinyl chloride in inspired air is also carcinogenic to rats. This indicates the need for further evaluation, as soon as results of the oral studies on vinyl chloride become available.

(f) Percutaneous migration of vinyl chloride.
(g) Levels of vinyl chloride in food; estimated daily intake in children and adults.
(h) Interaction of vinyl chloride with food and drink components.
(i) Levels of vinyl chloride in PVC products (sheets, film).
(j) Levels of vinyl chloride in potable water and PVC tubing.

The vinyl chloride problem should be capable of solution without recourse to total abolition of PVC manufacture and usage, but it was recognized that the latter policy might be adopted in some countries as a matter of expediency. Should this occur, there would be considerable risk to other plastics materials. It is obviously desirable that the co-operation of all interested parties be attained to facilitate and expedite the resolution of this problem.

THE WINDING MONOSODIUM GLUTAMATE TRAIL

The treatment of newborn rats with monosodium glutamate (MSG) in intragastric doses of 1·25, 2·5 or 5 g/kg/day for 5-day periods has been shown to induce behavioural defects, particularly in discrimination aptitude, and to reduce spontaneous motor activity, most strikingly in animals that had received the higher doses (*Cited in F.C.T.* 1973, **11,** 686). Subsequently, Johnston (*Biochem. Pharmac.* 1973, **22,** 137) reported that convulsions were produced by ip injection of neutral aqueous solutions of MSG, aspartate, methylaspartate, homocysteate, β-*N*-oxalyl-L-α,β-diaminopropionate or ibotenate into 10-day-old rats in doses up to 20 mmols/kg body weight. L-Aspartate and D- and L-glutamate proved convulsant only at the highest test dose of 20 mmols/kg and after about 30 minutes, whereas the other amino acids were considerably more potent in this respect. The convulsant activities of these compounds in neonatal rats were roughly in line with their relative abilities to excite feline central neurones, which suggested that the same basic mechanism was involved in the two effects. Several of these amino acids, in addition to MSG, are known to produce neuronal damage in the hypothalamus of infant mice after sc injection.

A paper by Olney *et al.* (*New Engl. J. Med.* 1973, **289,** 391) indicates that glutamic, aspartic and cysteic acids present in casein hydrolysates intended for iv therapy may pose a hazard. Parenteral feeding of such solutions to surgical and paediatric patients, including premature infants, is apparently increasing. Olney *et al.* (*loc. cit.*) gave casein hydrolysates sc to 10-day-old mice in a single dose of 20–100 ml/kg and found that these, like an injection of a fibrin hydrolysate in a dose of 80–100 ml/kg, induced acute degeneration of neurons in the developing hypothalamus. The combined concentrations of acidic amino acids were 2460 μmols/100 ml in the casein hydrolysate and 1130 μmols/100 ml in the fibrin hydrolysate. The hypothalamic lesions appeared to be identical with those induced by oral or parenteral treatment with glutamic, aspartic or cysteic acid, and control injections with an amino acid mixture free of these three compounds caused no hypothalamic damage. On the other hand, Arthur *et al.* (*Proc. Soc. exp. Biol. Med.* 1973, **144,** 34) found that, in weanling mice, daily sc injections of 1 g MSG/kg or 0·92 g monosodium aspartate/kg for up to 21 days had no significant effect upon the activities of glutamic dehydrogenase or glutamic–oxalacetic and glutamic–pyruvic transaminases in brain or liver. The same doses given to newborn mice, however, caused a two- or three-fold increase in the activity of these enzymes in both brain and liver. Evidently weanling mice are able to metabolize relatively large quantities of these amino acids, although newborn mice cannot.

We have seen that, in mice, only glial cells and not neurones are damaged by oral administration of MSG, and that in monkeys, whose central nervous system is fully myelinated at birth, even large doses of MSG fail to have any such effect (*Cited in F.C.T.* 1972, **10,** 585). Chi-Pang Wen *et al.* (*Am. J. clin. Nutr.* 1973, **26,** 803) studied the effects of dietary supplements of MSG on infant squirrel monkeys, weanling rats and suckling mice. Feeding 4·8–16·7% MSG to the monkeys for 9 weeks failed to induce hypothalamic or retinal lesions. These authors also detected no adverse effect when they fed a diet containing 9·1% MSG to infant cynomolgus and bushbaby monkeys for 1 year. Weanling rats fed 20 or 40% MSG for 5 weeks showed some depression of growth, which was attributed to the excessive sodium intake rather than to glutamate as such. Infant mice given 2, 6 or 9 g MSG/kg on days 6–10 of life had developed no hypothalamic lesions when killed on day 11. In a similar experiment not terminated at day 11, about one-third of the treated mice died within 30 days, apparently from the effect of the high-sodium diet and/or the high osmolarity of the MSG solution. The ultimate survivors failed to become obese or hyperactive within 1 year. This study thus supports the view that considerable amounts of MSG appear to be harmless when ingested as part of an otherwise normal diet.

Some data regarding the absorption and distribution of MSG, when given alone or in conjunction with an infant food formula, have been given by Stegink *et al.* (*J. Nutr.* 1973, **103,** 1138). In fasted 3-day-old pigs given 0·01, 0·1 or 1·0 g MSG/kg by gavage, plasma glutamate levels reached a maximum 15–30 minutes after administration of MSG given alone in solution, or 90–120 minutes after administration in infant food. The maximum level ultimately reached was not affected by the vehicle. Plasma amino acid levels did not differ significantly between pigs given 0·01 g MSG/kg and the controls, but 0·1 g/kg produced slight increases in plasma glutamate and aspartate at times when absorption of MSG was at its maximum. With 1·0 g MSG/kg, there were marked increases in plasma glutamate, aspartate and alanine. With the two lower levels of MSG (the only two studied in this connexion), there were no significant changes in free amino acid concentrations in brain, muscle or cerebrospinal fluid. The considerable metabolism of glutamate in the liver was confirmed by the demonstration that levels of glutamate in portal blood were five times higher than those in peripheral blood, even in control animals.

Further studies of MSG metabolism in newborn pigs by Stegink *et al.* (*ibid* 1973, **103,** 1146) followed the distribution of radioactivity after administration of U-^{14}C-labelled MSG in a dose of 1 g/kg by gavage. Radioactivity promptly appeared in plasma glutamate, arginine, aspartate, glutamine, alanine, ornithine, citrulline, urea and two ninhydrin-negative metabolites, subsequently found to be glucose and lactate. Glucose, glutamate and lactate between them accounted for 65–80% of the total activity. Very small amounts of radioactivity appeared in pyruvate and α-ketoglutarate, but there was no significant amount in plasma succinate, pyrrolidone carboxylate, malate, citrate or oxalacetate. Moreover, it appeared that no labelled glutamate or aspartate entered the cerebrospinal fluid, although substantial quantities of labelled glutamine, glucose, lactate and urea were detected here.

It appears from this latest collection of studies that while there is still little evidence to suggest that oral administration of MSG, even in considerable amounts, might give rise to lesions of the central nervous system, there may be a case for looking further into the question of parenteral nutrients destined for injection into premature infants. In considering the work on such preparations, however, it is important to bear in mind that myelination, which is thought to contribute to the blood-brain barrier, occurs at a much earlier

stage of development in man than in mice (*Cited in F.C.T.* 1972, **10,** 585), so that results obtained in newborn mice must be interpreted with caution.

[P. Cooper – BIBRA]

ALCOHOL MUSCLES IN

Alcoholic cardiomyopathy

The question of the effects of alcohol on the heart sprang into prominence some 7 years ago in connexion with several outbreaks of fatal heart disease among heavy beer drinkers. This particular syndrome was associated with a combination of factors, including the consumption of beer to which cobalt had been added to improve the foaming properties (*Cited in F.C.T.* 1972, **10,** 99), and was characterized by certain features not found in other types of cardiomyopathy, such as that induced by high alcohol consumption alone.

Though less spectacular, the latter type of cardiomyopathy is no less important. Reviewing the problem of alcoholism in Australia, where excessive drinking is held to be responsible for 3% of all deaths, Laurie (*Med. J. Aust.* 1971, **1,** 1224) suggested that cardiomyopathy due to a very high intake of alcohol was probably an underestimated factor in morbidity, since it could easily go undetected in autopsies following sudden death. Alcoholic cardiomyopathy is a common, though not invariable, complication of heavy or prolonged drinking and, according to Laurie (*loc. cit.*), this is attributable in most cases to a direct toxic action on the myocardium, nutritional deficiency and cobalt toxicity being involved only in relatively rare and special cases. In some instances, ethanol produces a microscopically visible fibrosis affecting the left and sometimes also the right ventricle, and the myocardium becomes oedematous and vacuolated, with fatty degeneration and an increase in interstitial tissue (Laurie, *loc. cit.*).

The cause of alcoholic cardiomyopathy is disputed. Burch & Giles (*Am. J. Med.* 1971, **50,** 141) believe that it is produced by chronic damage to the myocardium brought about by important changes in metabolism. Electron microscopy of affected myocardial tissue has revealed marked mitochondrial swelling with fragmentation of the cristae, swelling of the sarcoplasmic reticulum and disruption of myofibrils. Increased numbers of lysosomes and granules of lipofuscin and other pigments are distributed throughout the myocardium, and histochemical studies have demonstrated deposits of lipids, particularly triglycerides, within the myocardial fibres together with severe enzyme depletion. While such toxic changes follow chronic alcohol abuse in the absence of nutritional deficiencies (Burch & Giles, *loc. cit.*), they constitute a non-specific response which is found in various other diseases. In its early stages alcoholic cardiomyopathy is reversible, but when advanced it can only be treated by prolonged bed rest in conjunction with conventional therapy for congestive heart failure.

Shanoff (*Can. med. Ass. J.* 1972, **106,** 55), who considered that relatively few heavy chronic drinkers developed cardiomyopathy, suggested that this lesion was explicable on the grounds of an individual susceptibility. The disease process starts with severe damage to heart muscle produced by high ethanol concentrations, and this is followed by marked heart dilatation and an increase in wall tension, which the ventricles are unable to overcome. Thus, according to Shanoff (*loc. cit.*), it is the inadequacy of the usual compensatory mechanism of hypertrophy that leads to progressive and intractable ventricular failure in such individuals, most of them middle-aged men who have been drinking heavily for years.

Certainly coronary atherosclerosis does not appear to be involved in the pathology of this condition (Laurie, *loc. cit.*). Hognestad & Teisberg (*Acta path. microbiol. scand.* 1973, **81**A, 315) examined hearts from 35 alcoholic and 13 non-alcoholic traffic casualties and found that both groups exhibited a similar degree of coronary atherosclerosis. In two-thirds of the hearts from the alcoholics there were changes explicable only on the basis of chronic ethanol intoxication. A combination of cardiac hypertrophy and subendothelial, interstitial and perivascular fibrosis, together with the presence of chronic inflammatory cells, is indicative of alcoholic heart disease, which these workers again consider to be commoner than is generally supposed.

Studies of the symptoms of chronic alcoholism in 107 men, more than half of them saké drinkers (Koide *et al. Jap. Heart J.* 1972, **13**, 418), showed palpitation or shortness of breath in 49, an abnormal electrocardiogram (ECG) in 65 and orthostatic dizziness or syncope in 34. Cardiac enlargement occurred exclusively in those patients whose heavy drinking had extended over a period of 10 years or longer, while ECG abnormalities were most often associated with heavy drinking for at least 5 years. Bayer & Jost (*Dt. med. Wschr.* 1972, **97**, 722) found cardiomegaly with progressive left and right ventricular failure in six middle-aged men who had consumed large quantities of alcohol for years. Two of them, who died shortly after the diagnosis had been made, showed hypertrophy involving the atria and ventricles, with essentially normal heart valves and coronary arteries.

In a series of post-mortem examinations of 97 alcoholic patients, Schenk & Cohen (*Pathologia Microbiol.* 1970, **35**, 96) reported heart weights exceeding 300 g in 90 and exceeding 500 g in 29 (normal 270 g). Only six hearts showed marked coronary atherosclerosis and none had valvular or other structural abnormalities. Only three of the patients had been hypertensive. Diffuse interstitial fibrosis, apparent microscopically in 87 of the 97 hearts, did not seem to be the result of vascular constriction or occlusion. No correlation could be established between the cardiomyopathy and the type and degree of fatty degeneration and cirrhosis observed in the liver. However, cardiomyopathy is not necessarily associated with cardiomegaly, for Asokan *et al.* (*Am. Heart J.* 1972, **84**, 13) described nine alcoholics who had a mild impairment of heart function but who showed no increase in cardiothoracic ratio when given chest X-rays and whose ECG was normal. Impairment in these men took the form of an elevated mean left ventricular diastolic pressure, a low mean cardiac output, and a depression in myocardial contractility.

Experiments reviewed by Gould (*ibid* 1970, **79**, 422) have shown that chronic alcoholics without clinical, haemodynamic or biochemical evidence of heart disease may show a consistently negative myocardial balance of isocitric dehydrogenase and malic dehydrogenase. This evidence suggests that repeated bouts of alcoholism may permanently alter metabolic pathways or the permeability of mitochondrial membranes in the heart. This possibility is favoured by the observation that chronic alcoholics without evident heart disease sometimes make an abnormal ventricular response to exercise by reason of impaired ventricular metabolism and contractility. There is no evidence, says Gould (*loc. cit.*), that ethanol has any therapeutic value in treating angina pectoris, apart from its sedating effect.

Animal experiments

The results of feeding studies in which ethanol was given to animals as a substantial part of their caloric intake have not been very helpful in determining the nature of the cardiomyopathic lesion. When Hall & Rowlands (*Am. J. Path.* 1970, **60**, 153) gave otherwise well-nourished rats a diet in which 33% of the caloric intake was replaced with ethanol for

up to 14 weeks, fatty changes appeared in the livers of the treated rats but there was no difference in myocardial microstructure between these rats and a control group. Thus far, the evidence points to a lack of any direct toxic effect of ethanol on the heart of the rat. Similar results have been reported by Yamashita (*Jap. Heart J.* 1971, **12,** 242) who gave 15% (v/v) saké, 35% shōchū, 39% whisky or 20 or 40% ethanol to rats daily by stomach tube in a dose of 15 ml/kg for 11 weeks. The animals failed to show any heart enlargement, histological changes in the myocardium or ECG abnormalities by the end of the experiment, in which the intake of ethanol was estimated to be equivalent to about 1 litre daily by an adult man for about 2·5 years. However, Gvozdják *et al.* (*Biochem. Pharmac.* 1973, **22,** 1807) found that daily ip administration of 2·5 g ethanol/kg to rats for 10 weeks retarded growth, decreased myocardial glycogen content and depressed respiration and glycolytic activity in the myocardial-tissue homogenate. The mitochondria showed an insignificant decrease (8·7%) in respiration compared with controls, but their respiratory control index (a measure of the degree of oxidative phosphorylation) fell significantly (by 28·2%). Heart weight fell in parallel with body weight. Despite the considerable alterations in myocardial glycolysis and oxidative phosphorylation, no morphological changes were detected in the heart, either by histological examination or by electron microscopy. The authors suggest that the relatively brief period (about 5 hours daily), during which ethanol remained in the blood, myocardium and liver, militated against any chronic effect. In dogs that consumed 100 ml ethanol daily for 14 wk in the form of 400 ml of a 25% solution added to the food and water consumed over each 24-hour period, the blood alcohol levels averaged 180 mg/100 ml, with a peak of 240 mg/100 ml after ingestion of food (Pachinger *et al. J. clin. Invest.* 1973, **52,** 2690). Ethanol was withheld for 2 days before studies of mitochondrial function and myocardial contractility were carried out. There was again a decrease in mitochondrial oxygen consumption and in the respiratory control index, together with a reduction in intramitochondrial isocitrate dehydrogenase and myocardial high-energy phosphate. However, total myocardial blood flow, cardiac output and myocardial oxygen consumption were unaffected by the ethanol intake and no significant change in cardiac contractility was demonstrated under conditions of an increased cardiac load. The observations of Burch *et al.* (*Johns Hopkins Med. J.* 1971, **129,** 130; cited from *British Medical Journal* 1972, **2,** 247) on mice exposed to an unrestricted intake of various forms of ethanol for 6 months have shown that focal myocardial damage involving hydropic degeneration, loss of striation in some muscle fibres, interstitial oedema and occasional round-cell infiltration and fibroblastic proliferation may accompany the persistent and excessive consumption of alcohol. Electron microscopy showed accumulation of lipid, fragmentation of myofibrils and other changes in 56% of the treated rats, while 35% showed histochemical alterations.

Skeletal muscle lesions

Turning now to the possible effects of alcohol consumption on skeletal muscles, Mayer (*Ann. N.Y. Acad. Sci.* 1973, **215,** 370) investigated in rats the possibility that ethanol damages the muscle membrane and eventually the whole muscle fibre. The animals were fed a diet modified by isocaloric substitution of 36% of carbohydrate by ethanol. None of the rats observed up to 36 weeks exhibited limb weakness. The resting membrane potential in the extensor digitorum longus muscle was slightly reduced in a few. Rats taking ethanol for 6–36 weeks showed an increase in sarcolemmal nuclei in occasional muscle fibres, but histochemical staining revealed no abnormalities. The possibility that ethanol

consumption may alter the ultrastructure and membrane function of muscle cannot be discounted, but its progression to clinical myopathy remains in doubt on the strength of this evidence.

Nevertheless, acute alcoholic myopathy is a striking, if relatively rare, syndrome usually associated with a prolonged bout of heavy drinking in a chronic alcoholic. Slowly developing weakness and wasting of proximal limb muscles has also been described in chronic alcoholics (*Lancet* 1971, **i,** 1171) and biopsy specimens from such patients have indicated myopathy. However, neuropathy—a common complication of chronic alcoholism —may be the underlying factor in these chronic muscular disturbances, although there is little room for doubt about the real existence of the state of acute alcoholic myopathy. Sheremata & Sherwin (*Dis. nerv. Syst.* 1972, **33,** 136) have described complete urinary retention in a 43-year-old man who had been drinking heavily on occasion over 26 years, and particularly over the past 6 months. Further examination showed mild weakness of the extensors of the wrist and abductors of the fingers, and marked weakness of the extensors and flexors of the lower limbs. He was unable to walk. Muscle strength was much improved by a regimen of a high-protein, high-calorie diet with iron, thiamine and multivitamin supplementation. It was deduced that the lesion responsible for this clinical picture was likely to be in the spinal cord. Curran & Wetmore (*Dis. nerv. Syst.* 1972, **33,** 19) have reported the case of another middle-aged man, who had suffered recurrent episodes of diffuse muscle pain, tenderness and weakness and who also had a history of congestive heart failure, which was considered secondary to cardiomyopathy. He had been a heavy consumer of alcohol for many years. Biopsy specimens of muscle showed necrosis in scattered individual fibres, some 5% of the fibres being affected altogether, and the serum activities of creatine phosphokinase (CPK), lactic dehydrogenase and glutamic–oxalacetic transaminase were raised. A provocative test with ethanol after his recovery caused a prompt recurrence of muscle pain and a rise in CPK activity.

Experiments in three non-alcoholic volunteers who lived for 28 days on a diet in which ethanol accounted for 42% of the total calories showed that a rise in serum CPK, indicative of muscle damage, occurred during ethanol ingestion but reverted to normal within 2 weeks of a return to normal diet (Song & Rubin, *Science, N.Y.* 1971, **175,** 327). Biopsy specimens of muscle showed no light-microscopic abnormalities, but electron microscopy at the end of the experiment demonstrated intracellular oedema, widened interfibrillar spaces containing glycogen, lipid droplets, deranged elements of sarcoplasmic reticulum and irregular and sometimes enlarged mitochondria. These authors suggest that similar changes may occur in cardiac muscle and play a role in alcoholic cardiomyopathy. In electron-microscopic studies of biopsy specimens of skeletal muscle from 15 patients with muscular weakness, Fisher *et al.* (*Am. J. med. Sci.* 1971, **261,** 85) found mitochondrial inclusions and other mitochondrial changes in nine cases. Inclusions were not found in the other six, but changes involving the cristae produced a mosaic-like pattern. Ten of these 15 patients gave a history of chronic alcoholism. No consistent rise in serum CPK was found in these patients, but their alcohol consumption immediately prior to and during the study is not reported.

Conspicuous mitochondrial abnormalities were also reported in muscle biopsies from cases of acute alcoholic myopathy studied by Martinez *et al.* (*J. neurol. Sci.* 1973, **20,** 245). Patchy myofibrillar degeneration and regeneration and interstitial inflammation in the biopsy tissues were accompanied by a patchy loss of oxidative enzyme activity in some fibres, and electron microscopy revealed partial or total dissolution of mitochondria with disor-

ganization and swelling of the cristae. Inclusions were present in some of the degenerating mitochondria. Martinez *et al.* (*loc. cit.*) identify these changes as representing a non-specific response to a variety of non-related agents that damage skeletal muscle.

There are difficulties in diagnosing chronic alcholic myopathy, as Shin Joong Oh (*Sth med. J., Nashville* 1972, **65,** 449) has pointed out. He recounts the case of a 30-year-old man, a heavy drinker for many years, who developed generalized muscle weakness progressing to complete quadriplegia. He was found to have a blood-lead concentration of 97 μg/100 ml. When this was reduced by versenate treatment his improvement was still only slight. His marked disability was attributed to subclinical neuropathy and myopathy brought about by alcoholism combined with an aggravation of the neuropathic effects by lead intoxication, which was probably the result of consumption of 'moonshine' whisky.

Myopathy is a relatively recent addition to the many sins of alcohol, but it seems probable that we shall be hearing more about it in the future.

[P. Cooper—BIBRA]

RAPESEED OIL AND ERUCIC ACID

In several animal species the feeding of rapeseed oil (RSO) as a major source of calories has led to reports of fatty deposition in heart muscle and associated myocardial lesions. These developments have been attributed to the relatively high content of erucic acid (EA) in the oil (*Cited in F.C.T.* 1974, **12,** 255). Work has continued on the biochemical and ultrastructural effects of RSO and EA in experimental animals and much of this has served to confirm the earlier studies.

Pipy *et al.* (*J. Physiol., Paris* 1972, **64,** 439) have examined the effects of feeding 20% RSO (containing only 50% EA), arachis oil (as a control) or canbra oil (containing only 0·5% EA) to rats for 12 weeks. RSO caused a clear reduction in growth rate in comparison with the other two oils. RSO and canbra oils had opposing effects on the reticulo-endothelial system, the former causing an increase in phagocytic activity and the latter a decrease. An increase in plasma triglycerides was recorded in rats given RSO but not in those given canbra oil. Neither treatment affected the accumulation of heart lipids after 12 weeks, although both increased the myocardial level of glucosamine, a change generally indicative of incipient fibrosis.

Another French group (Dallocchio *et al. C. r. Séanc. Soc. Biol.* 1973, **167,** 1122) have reported the results of feeding rabbits on diets containing these same three oils, in each case at a level of 15%. The test diets were given for 9 weeks and then for 3-week periods separated by similar periods on a normal diet, for an overall period of 10 months. Plasma cholesterol showed a significant rise in the test groups compared with the normal-diet controls. The rise was more marked as the proportion of long-chain fatty acids in the oil increased, the maxima being 0·8, 0·6 and 0·5 g/litre for RSO, canbra oil and arachis oil, respectively, compared with 0·4 g/litre for the controls. Myocardial fibrosis was noted in 72, 50 and 33%, respectively, of the animals given these three oils, compared with a control incidence of 8%. No correlation could be noted between the degree of hypercholesterolaemia and the severity of the myocardial lesions, and it was clear that EA did not carry the entire responsibility for the myocardial damage induced by these diets.

Rocquelin *et al.* (*Annls Biol. Anim. Biochim. Biophys.* 1973, **13,** 587), who fed a similar concentration of each of the three oils to rats for up to 60 days, reported that RSO rapidly produced a myocardial accumulation of triglycerides rich in long-chain monoenes (EA and

gadoleic acid). This accumulation reached its maximum during the first week of the experiment, the time when cardiac deposition of lipid was shown to be rapid in other studies (*Cited in F.C.T.* 1974, **12,** 255). Thereafter the myocardial fat content gradually fell, although after 60 days EA still constituted 8·1% of the total lipids. It seems therefore that myocardial metabolism becomes adapted to an oil-rich diet. Myocardial lesions were again associated with the ingestion of canbra oil as well as of RSO. The cardiac phospholipids contained EA from day 3 of RSO feeding, but this phenomenon did not occur with canbra or arachis oil. Slinger *et al.* (*Nutr. Rep. Int.* 1973, **8,** 245) found that weanling rats fed a diet containing 20% RSO rich in EA developed myocardial fat deposits during the first 3–6 days of feeding. Fat deposition in the heart was accompanied by depletion of cardiac glycogen, a state which persisted throughout the period of RSO feeding, although soon after day 6 the fat deposits began to diminish again. Transfer of animals to a maize-oil diet after 3–6 days on the RSO diet resulted in the rapid restoration of cardiac glycogen levels, but in animals fed the RSO diet for 9 days a change to maize oil produced no rise in cardiac glycogen. It appears from these findings that glycogen may be able to take the place of fat as a cardiac energy source when the myocardial fatty oxidation processes have been impaired by EA.

In experiments reported by Abdellatif & Vles (*Nutr. Metabol.* 1973, **15,** 219), rats were fed for 24 weeks on a diet in which EA in the form of RSO or glyceryl trierucate accounted for 25% of total calories. With either dietary addition, cardiac lipidosis developed after 1 week, but this decreased again to leave a residual cellular fibrosis by week 24. Although EA was considered to be the main pathogenic fatty acid in RSO, it was again thought that other long-chain fatty acids probably played a minor role in the development of the lesion. EA at a level providing 4% of total calories induced only mild cardiac lipidosis after 1 week and only slight to moderate myocardial scars after 24 weeks. A dose-related cardiac lipidosis was observed in rats fed 5–30% of calories as RSO for 3 or 6 days, and the first myocardial changes were observed after 32 weeks on the lowest of these RSO levels. Rocquelin *et al.* (*Archs Mal. Coeur* 1973, **66,** 1085) have pointed out that similar cardiac effects have been observed in rats, pigs and many other animal species, and in another paper the same group has reviewed the many papers dealing with the pathological effects of RSO and canbra oil on the heart and other organs (*idem, Cah. Nut. Diét.* 1973, **8,** 103).

Jaillard *et al.* (*Nutr. Metabol.* 1973, **15,** 336) have reported that lipoprotein lipase present in preparations of both the heart muscle and epididymal fat of rats was capable of liberating EA from chylomicrons. In rats given glyceryl trierucate orally, heart muscle was found to contain a high concentration of EA compared with that in the kidney, a finding attributed by the authors to the relatively slow breakdown of EA in the heart muscle. Kramer (*Lipids* 1973, **8,** 641) found when feeding weanling rats with RSO containing different levels of EA for 16 weeks that oil rich in EA depressed growth but did not make any significant difference to the total lipids, lipid phosphorus or cholesterol content of the liver compared with oil relatively poor in EA. However, the level of incorporation of EA and eicosenoic (20:1) acid into total liver lipids, neutral lipids, phosphatidylethanolamine and phosphatidylcholine was found to be proportional to the concentration of those acids in the feed oil.

The possible role of mitochondrial impairment in the observed effects of EA has been considered (*Cited in F.C.T.* 1974, **12,** 255). The β-oxidation of EA, oleic acid and palmitic acid by isolated rat mitochondria has been studied by Swarttouw (*Biochim. Biophys. Acta* 1974, **337,** 13), using the 1-^{14}C-labelled acids. Long-chain acyl-CoA synthetase from rat-

heart mitochondria was found to convert EA much more slowly than it converted palmitic acid. *In vitro*, the transport of the erucoyl group across the mitochondrial inner membrane through the agency of carnitine palmitoyltransferase took place at only about 12–17% of the rate for the palmitoyl group, although this state of enzyme activity was not thought to limit the overall conversion rate. Mitochondrial preparations oxidized the synthesized ester L(-)-palmitoylcarnitine at a higher rate than L(-)-erucoylcarnitine. Both the decreased rate of β-oxidation and the weaker binding of EA to carrier proteins may account for the accumulation of EA in cardiac muscle.

Pinson & Padieu (*FEBS Lett.* 1974, **39**, 88) studied the oxidation of EA by beating heart cells in culture, using both [1-^{14}C]EA and [14-^{14}C]EA as tracers. It was found that $^{14}CO_2$ arising from [1-^{14}C]EA appeared without delay, whereas that from [14-^{14}C]EA had a latent period of more than 12 hours. Curves of CO_2 production from the carboxylic and C-14 positions proved to be nearly parallel, with a displacement of about 16 hours. A study of the distribution of fatty acids and the percentage of radioactivity in triglyceride-bound EA indicated that the EA chain was shortened to Δ-11-eicosenoic and oleic acids only, while a small proportion was lengthened to nevronic acid (24:1). Apparently EA cannot be oxidized by myocytes in culture except via prior conversion to Δ-11-eicosenoic and oleic acids, and only after some 16 hours is equilibrium reached between the respective rates of transformation and oxidation.

Previous work has indicated the poor digestibility of EA in fowls and some impairment of egg production in RSO-fed hens (*Cited in F.C.T.* 1974, **12**, 256). Further experiments were performed by Vogtmann *et al.* (*Nutr. Metabol.* 1973, **15**, 252) to compare the effects of feeding RSO of high and low EA content, soya-bean oil and lard. The digestibility of total fatty acids increased with increasing amounts of dietary oil or fat. When the test lipids were fed at a dietary level of 5%, digestibility was lowest for RSO of high EA content (85·5% utilization) and highest for soya-bean oil (90·3%), but at the 10 and 15% levels, high-EA RSO and canbra oil (low in EA) were utilized to a comparable degree and were better utilized than lard. Nevertheless, when chicks were fed RSO, their body-weight gain decreased as the percentage of RSO increased, an effect attributed to a decrease in food intake rather than to impaired utilization.

In an experiment to determine whether the feeding of RSO affected the performance of laying hens, Lall & Slinger (*Poult. Sci.* 1973, **52**, 1729) found that 10 or 20% of a high-EA RSO in the diet depressed egg production, egg weight, yolk weight and hatchability, when compared with the performance of hens fed tallow or maize oil. However, a sample of RSO of low EA content had no detrimental effect, supporting earlier indications that EA is the factor primarily responsible for RSO-impairment of reproductive function in fowls (*Cited in F.C.T.* 1974, **12**, 257). The yolks of eggs from hens fed RSO with a high EA content contained on average 0·6% EA, while those from birds on a low-EA oil contained 0·2% EA. The cholesterol content of the yolks was greater from RSO-fed hens than from hens fed a basal diet without added fat, but was lower in the RSO-fed birds than in those on maize oil.

In a similar vein, Leslie *et al.* (*Can. J. Anim. Sci.* 1973, **53**, 747) reported a significantly lower egg production from hens fed 10 or 20% RSO than from those fed a ground corn and soya-bean meal diet. Food consumption was reduced in birds given a diet containing 10% RSO, 20% full-fat rapeseed or 20% rapeseed meal, while the feeding of the first two diets also reduced egg size. Shell thickness was improved by feeding 3% RSO but adversely affected by the 10% RSO diet. No distinct flavour or odour was imparted by the

10% RSO diet, although eggs from birds given this diet were consistently rated lower by a taste panel.

The practical implications of these findings to the welfare and economics of the domestic hen may be relatively clear, but there remains the difficulty of interpreting most of the experimental studies in terms of their significance to man. Jaillard *et al.* (*loc. cit.*) reported a small study in which they detected no preferential extraction of EA by the human myocardium from circulating free fatty acids when they sampled blood from the coronary sinus and from the femoral artery 3 hours after injestion of purified RSO, containing 49% EA, in a dose of 1 ml/kg. In this study, the only fatty acid that did show evidence of a preferential absorption by the human heart was oleic acid.

Meanwhile, considerable effort has been directed towards the breeding of varieties of rapeseed low in EA, notably in Canada (Rakow & McGregor, *J. Am. Oil Chem. Soc.* 1973, **50,** 400), France (Vodovar *et al.*, *C. r. hebd. Séanc. Acad. Sci., Paris* 1974, **278,** 943) and Germany (Röbbelen, *Qualitas Pl. Mater. veg.* 1973, **23,** 221). In Canada—where the trends in the use of RSO in the domestic market may perhaps offer some indication of its potential in world markets—RSO is reported to be gradually displacing other, long-established, edible oils (McAnsh, *J. Am. Oil Chem. Soc.* 1973, **50,** 404). In 1971 it accounted for some 36% of the 205,000 metric tons of vegetable oils used in the manufacture of margarine, shortening and salad oils, while soya-bean oil accounted for 32%. Comparison of these figures with those for 1966, when the corresponding proportions were 25% for RSO and 38% for soya-bean oil, provides some evidence of the growing importance of RSO.

[P. Cooper—BIBRA]

THE ELUSIVE AETIOLOGY OF ANENCEPHALY

Attempts to rationalize the mass of available epidemiological data relating to the incidence of anencephaly in the human embryo have so far met with little success. A genetic aetiology for this ailment, discredited in recent years by the lack of concordance in the incidence of the disease among monozygotic twins, cannot be discounted completely, since these twins may differ in the cytoplasmic component which represents an alternative channel for the transmission of cellular anomalies (*Cited in F.C.T.* 1974, **12,** 775). Nevertheless, many workers have pointed to seasonal, national, regional, urban/rural and even social differences in susceptibility as a possible indication that some environmental agent is responsible for this ubiquitous ailment. The study of possible environmental causes seems to offer some promise, and since various investigations have given no reason to implicate an infective mechanism, attention has centred on the possible involvement of nutritional factors in the aetiology of anencephaly. Random selection of a suspected food item on the basis of limited epidemiological data is liable, however, to lead to grave misjudgements, since a particularly high intake of a specific foodstuff may merely be a reflection of a less obvious but more important difference in overall nutritional status.

Last year (*ibid* 1973, **11,** 1164) we reviewed a study of possible associations between neural-tube defects and various dietary components, and this received further attention at a recent Royal Society of Medicine meeting. Knox (*Proc. R. Soc. Med.* 1974, **67,** 355) compared official statistics on the quarterly dietary intakes of various foods by the population of England and Wales over a 7-year period with the quarterly incidence of anencephaly recorded for the period 5–9 months later. Among the large number of positive associations identified, the most striking implicated bread, cereals, ice-cream, canned peas and

a variety of cooked and cured meats. Other superimposed correlations led to the elimination of some apparent associations and examination of the biological plausibility of the remaining foods suggested that cooked meats were by far the most likely to contain an insidious toxin. The suspicion centred on the possible production of nitroso compounds in the meat, through interaction between the nitrite preservative and constituent amines. The problem posed by the presence of low levels of nitrosamines in food is an extremely complex one, and insufficient information is at present available on the occurrence of non-volatile nitrosamines in food and on the possible synthesis of nitroso compounds *in vivo*. Some of these alkylating agents can produce teratogenic effects at very high dose levels, although there seems to be no evidence of teratogenicity in the case of dimethylnitrosamine (DMN), the volatile nitrosamine that seems to have been most frequently identified in foods (DiPaolo, *Ann. N.Y. Acad. Sci.* 1969, **163,** 801).

Among 17 samples of canned, cured meat examined by Panalaks *et al.* (*J. Ass. off. analyt. Chem.* 1973, **56,** 621), four samples contained detectable levels of DMN (2–3 ppb; b = 10^9), while of 23 samples of corned beef products, five contained DMN above the detection limit, the highest level found being 12 ppb. In general, there was no correlation between DMN content and the levels of nitrite and nitrate in the meat. By way of comparison, it may also be pointed out that DMN (up to 9 ppb) has also been detected in several species of fish (Crosby *et al. Nature, Lond.* 1972, **238,** 342), but fish was not among the foods identified by Knox (*loc. cit.*) as showing some apparent correlation with the incidence of anencephaly. It must also be borne in mind that anencephaly, unlike many of the ills of modern man, has afflicted *Homo sapiens* for several thousand years at least (*Cited in F.C.T.* 1974, **12,** 775) and thus antedates the widespread use of nitrites as curing agents by some considerable period.

A recent comparison of the incidence of anencephaly in 25 major countries with the annual consumption of common foods and beverages (Fedrick, *Proc. R. Soc. Med.* 1974, **67,** 356) yielded a significant positive correlation only for the consumption of tea. There was no significant association with either bread or sugar as suggested in an earlier survey by Edwards (*Br. J. prev. soc. Med.* 1958, **12,** 115), while cooked meat was not among the foods specifically examined. On the basis of these findings, a retrospective study was undertaken, involving 558 women resident in England and Wales who had produced a still-born anencephalic infant and 2232 control women matched for age, parity, geographical location and date of delivery. These results suggested that the mothers of the anencephalics were significantly more likely ($P < 0{\cdot}001$) than the controls to drink three or more cups of tea each day, but only if they resided in "high or medium incidence" areas. Fedrick (*loc. cit.*) points out certain shortcomings in her work. One such defect is the time lag of up to 9 months between the birth of the child and the collection of data on maternal drinking habits, which were then assumed to have remained unchanged for some 12–18 months. She also stresses that the consumption of tea may be associated with other factors which have not been identified and comments that while an earlier study (*Cited in F.C.T.* 1973, **11,** 1164) indicated a significant positive correlation between tea consumption and neural-tube malformation, this was related to a parallel secular trend. Nevertheless, it is of some interest to view these findings on tea-drinking in the light of previous surveys that have suggested a possible link between the softness of the water supply and the incidence of anencephaly in specific localities (Fedrick *Nature, Lond.* 1970, **227,** 176; Lowe *et al. Br. med. J.* 1971, **ii,** 357).

The speciously attractive proposition that heavy metals in soft water may contribute

to the aetiology of anencephaly seems unrealistic in the face of both epidemiological evidence and data from animal experimentation. While it can be argued that soft water is more liable than hard to erode lead piping, no positive correlation can be deduced from comparing the annual perinatal death rate from anencephaly in the period 1963–1967 in 36 UK cities, as listed by Lowe *et al.* (*loc. cit.*), with the contamination of drinking-water by lead and other heavy metals in the same cities (Read & Tolley, *Vitalstoffe* 1968, **13**, 250). Moreover, although Clegg (*Fd Cosmet. Toxicol.* 1971, **9**, 195) has drawn attention to several reports suggesting that severe environmental exposure of pregnant women to lead may result in abortion or neurological symptoms, the lead intake in these cases was far in excess of possible dietary exposure. Relatively little experimental work has been done on the possible teratogenic effects of orally administered lead, but recently Fournier & Rosenberg (*Proceedings of an International Symposium on Environmental Health Aspects of Lead*, Amsterdam, 2–6 October 1972, p. 287) reported that rats and rabbits given lead acetate orally in doses of 1 or 2 mg/kg/day on days 5–12 of pregnancy or of 1 or 2 mg/kg/day for 8 weeks before mating showed an incidence of congenital malformations no higher than that in controls. Neither were developmental abnormalities induced by the feeding of lead in the diet to ruminants (Shupe *et al. J. Am. vet. med. Ass.* 1967, **151**, 198).

Altogether, despite the determined efforts of many workers, very little progress has been made in the search for a link between some environmental factor and anencephaly. Considering the social and economic implications of this type of birth defect, such efforts are to be encouraged, but one must always be aware of the need to evaluate carefully the results obtained to prevent any precipitate action which might have serious repercussions.

[J. J.-P. Drake—BIBRA]

MORE DETAILS ON NITROGEN DIOXIDE INHALATION

Nitrogen dioxide (NO_2) is a widespread atmospheric pollutant, which has been shown to induce pulmonary damage in rats (*Cited in F.C.T.* 1970, **8**, 219) and dogs (*ibid* 1972, **10**, 727) at exposure levels of only a few parts per million. Higher levels (50–100 ppm) have been reported to have clearly adverse effects on the human lung and some workers have drawn attention to the possibility that persistent exposure to very low levels in the atmosphere may eventually have some deleterious effect in man (*ibid* 1970, **8**, 220). Recent work has extended existing knowledge on the pulmonary effects of NO_2 inhalation, particularly in rats.

Pulmonary changes

Stephens *et al.* (*Archs envir. Hlth* 1972, **24**, 160) have exposed young rats to 17 ppm NO_2 for up to 43 days, and to 2 ppm NO_2 for up to 12 months. The higher concentration, but not the lower, retarded growth and caused yellowing of the animals' coats. After a few weeks, rats exposed to 17 ppm NO_2 had enlarged lungs and a greater dead-space than controls. Alveolar engorgement was evident at 2 hours and there was significant loss of cilia from the epithelial cells at 8 hours. Cell hypertrophy began at 48 hours. Injury to the tissues adjoining the terminal bronchioles included sloughing of type I pneumocytes and exposure of the basement membrane, followed by the thickening of the air-blood barrier with repair cells of a low cuboidal type. At 2 ppm, NO_2 produced hypertrophy of ciliated cells of the terminal bronchioles, with cloudy swelling and the disappearance of many cilia.

By day 7, the number of ciliated cells was greatly reduced and the epithelial surface was made uneven by patches of hyperplasia. By day 14 cilia had reappeared, though in reduced numbers, and after 21 days substantial recovery was evident.

In experiments reported by Freeman *et al.* (*Am. Rev. resp. Dis.* 1969, **100,** 662) rats were exposed to 15 ppm NO_2 for 1–20 weeks, and removed from further exposure for up to 52 weeks. Lung weights of exposed animals were higher than those of controls at two stages of the study. The first increase was due to hypertrophy of the bronchiolar and adjacent alveolar epithelium, and this tended to return to normal during the recovery phase. Then at about week 72, long after cessation of NO_2 exposure, an anomalous increase in the lung weight of the ageing rats was associated with an increase in the staining capacity of collagen and elastic tissue in the alveolar parenchyma. In rats given the longer exposures, some fibrosis of the terminal bronchioles and breaks in the continuity of the altered elastic tissue led to an apparent reduction in lung elasticity. Parkinson & Stephens (*Envir. Res.* 1973, **6,** 37) performed scanning electron microscopy of the distal airways and adjacent regions of lungs derived from rats exposed to 15 ppm NO_2 for 1–7 days, and showed that initially NO_2 induced a loss of cilia and produced an overall uniformity of surface in the terminal bronchioles by reducing the difference in size between ciliated and supporting cells. In the region of the proximal alveolar ducts adjacent to the terminal bronchioles, type 1 cells were replaced to a marked extent by others covered with microvilli. Exposure to NO_2 also caused an increase in the numbers of macrophages and the amount of alveolar debris.

The recovery process

Studies using tritiated thymidine (^{3}H-TdR) have been carried out to determine the nature of the cellular repair process which follows exposure to NO_2. Evans *et al.* (*Am. J. Path.* 1973, **70,** 175) have described their observations on male rats exposed to 15–17 ppm NO_2 for 48 hours and then given an ip injection of ^{3}H-TdR as a label for regenerating cells. In NO_2-exposed rats, they found a large increase in the number of type 2 cells (cuboidal, vacuolated cells lying on the alveolar surface) taking up the label. All the cells examined by electron autoradiography during mitosis showed lamellar bodies, mitochondria, which were normal in appearance, and microvilli on their free surface; they were attached by at least one edge to the basement membrane. The labelled type 2 cells in the alveoli of exposed rats appeared to be randomly distributed throughout the alveoli, peripheral to the opening of terminal bronchioles. These type 2 cells are capable of dividing into sister cells, which, in turn, are transformed into type 1 cells, and the renewal of alveolar epithelium appears to involve the production of type 1 cells in this way. In a further stage of this work (*idem, ibid* 1973, **70,** 199), the duration of the cell cycle of dividing alveolar macrophages in young rats exposed to 15–17 ppm NO_2 for 48 hours was estimated to be very short (9·2 hours). This evidence is compatible with an interpretation of cell renewal in which cells derived from the bone marrow enter the alveoli from the vascular system and become macrophages, dividing and thus increasing in number until they are eventually extruded from the alveoli.

Further studies were undertaken in male rats exposed to 2 ppm NO_2 for up to 360 days, or to 17 ppm NO_2 for 7 days (*idem, Archs envir. Hlth* 1972, **24,** 180). After long exposure to 2 ppm NO_2, the alveoli at the distal ends of terminal bronchioles showed no increase in ^{3}H-TdR-labelled cells, whereas exposure to 17 ppm NO_2 for 48–72 hours provoked an increase in the number of labelled cells with type 2 characteristics. After exposure to

17 ppm NO_2 for 96 hours, the number of labelled cells significantly diminished, until by day 5 it had reached normal levels, leaving alveolar tissue of increased cellularity. There was a small increase in labelled cells after exposure to 2 ppm NO_2 for up to 72 hours, but this subsequently decreased to normal. Other observations by this group (*idem, Archs intern. Med.* 1971, **128,** 57) in young rats exposed to 17 ppm NO_2 have demonstrated that, whereas ^{3}H-TdR labelling of type 2 cells of the terminal bronchioles and alveolar walls was increased some fourfold during the first few days, there was no parallel effect on leucocytes or endothelial and interstitial cells within the alveolar walls. Enhanced cell renewal after NO_2 exposure may therefore result from direct stimulation by NO_2 at some stage of cell division, or may be a secondary response to cellular injury, in which the compensatory increase in cell renewal exceeds cell loss for a short time and then stabilizes.

Effect on mucociliary clearance

A technique using droplets of ^{99m}Tc-labelled sodium pertechnetate solution to indicate ciliary activity was used by Giordano & Morrow (*Archs envir. Hlth.* 1972, **25,** 443) to determine the effect of exposure to 6 ppm NO_2 for 6 weeks on mucociliary clearance in the lungs of rats. Measurements used to follow the rate of movement of the test droplets were taken as criteria of altered ciliary function, which was significantly depressed in most exposed animals although the effect was reversible within 7 days. No abnormality of the airways, apart from some oedema and vascular congestion in the alveolar areas, was apparent in these animals. It is suggested that the action of NO_2 on mucociliary movement may derive from an effect on the energy source of the cilia or on the composition of the mucous blanket. Sherwin & Carlson (*ibid* 1973, **27,** 90) have demonstrated that exposure of guinea-pigs to 0·4 ppm NO_2 for 1 week raises the protein content of the mucus obtained by lung lavage, but it is not clear whether protein leakage from the capillary bed or increased cell turnover is primarily responsible for this effect; both factors appear to be involved.

Milanesi *et al.* (*ibid* 1972, **25,** 301) have shown that the exposure of rat-lung homogenates to NO_2 does not significantly alter the binding state or specific free activity of the proteinase, cathepsin D, found in lysosomes. The surfactant properties of lung lecithin appear to be altered by exposure to NO_2, either by the direct action of the gas or by contamination with components of injured lung tissue (Williams *et al. Archs intern. Med.* 1971, **128,** 101). Exposure of rats to 15 ppm NO_2 for 28 days significantly increased the total phospholipids and lecithin in the alveolar washings, but did not alter the relative proportion of lecithin to other phospholipid or the surfactant properties of the washings. However, when Arner & Rhoades (*Archs envir. Hlth* 1973, **26,** 156) exposed rats to 2·9 ppm NO_2 for 24 hours daily on 5 days/week for 9 months, they found that the wet weight of the lungs was increased by 12·7%, lung compliance was reduced by 13% and the surfactant properties of the lung washings were significantly reduced. Lungs from exposed animals showed an 8·7% diminution in lipid content and a lower percentage of total saturated phospholipid fatty acids, a reduction attributable almost entirely to a fall in palmitate content. It seems, therefore, that some at least of the effects of subacute NO_2 exposure may result from alterations in pulmonary lipid metabolism.

Effect on resistance to infection

Henry *et al.* (*ibid* 1970, **20,** 566) reported that squirrel monkeys exposed to NO_2 continuously at a level of 10 ppm for 1 month or 5 ppm for 2 months showed an increased

susceptibility to aerosols of *Klebsiella pneumoniae*, as indicated by a raised mortality rate. Physiological effects of exposure to 10 ppm NO_2 included an increase in minute volume due to increases in both tidal volume and respiratory rate, while 5 ppm NO_2 depressed tidal volume gradually but did not affect minute volume because of a compensatory rise in respiration rate. After the *K. pneumoniae* challenge, the minute volumes of exposed animals decreased while those of controls increased. Clearance of inhaled bacteria from the lungs of NO_2-exposed monkeys was slower than that from controls. When challenged with influenza virus 24 hours before exposure to 10 ppm NO_2, all monkeys died within 3 days, while the controls developed only a non-fatal infection. When exposure to 5 ppm NO_2 occurred 24 hours after the virus challenge, one of the three monkeys died. On the other hand, Fenters *et al.* (*ibid* 1973, **27,** 85) found that squirrel monkeys exposed to 1 ppm NO_2 for 493 days and challenged five times during this period with a monkey-adapted influenza virus developed antibody within 21 days of the first inoculation, achieving serum neutralization titres ranging from 1:16 to 1:196, whereas only one control monkey showed a response at this time. By day 41 after this infectious challenge, the mean titre for the four NO_2-exposed monkeys was 11 times higher than that for the controls, which produced no marked antibody response until 105 days after the initial virus challenge. In monkeys exposed to NO_2 and the virus, slight emphysema and thickening of the bronchial and bronchiolar epithelium appeared, but no such change was seen in either group exposed to one of these agents alone.

In a study of mice exposed to various levels of NO_2 between 1 and 15 ppm for 17 hours before or 4 hours after inhalation of aerosols of ^{32}P-labelled *Staphylococcus aureus*, Goldstein *et al.* (*ibid* 1973, **26,** 202) found that exposure to 7 ppm NO_2 after or 2·3 ppm NO_2 before infection adversely affected the intrapulmonary bactericidal defence mechanism. Since 2·3 ppm lies only slightly above ambient atmospheric concentrations of NO_2, these findings suggest that human populations may incur an increased risk of pulmonary infection by environmental exposure to traces of NO_2. Acton & Myrvik (*ibid* 1972, **24,** 48) have reported that rabbits exposed to 15–50 ppm NO_2 for 3 hours yielded alveolar cells with a reduced capacity to develop virus-induced resistance and to phagocytize BCG vaccine. Measurable impairment of phagocytosis and virus-induced resistance occurred at a threshold of about 15 ppm NO_2. On the other hand, in preliminary studies, exposure to 50 ppm NO_2 for 3 hours failed to bring about any significant modification of the rate of protein or RNA synthesis in resting alveolar cells, which appear to be resistant to brief exposures to high concentrations of NO_2. This level of exposure did, however, promote oxygen consumption and glucose utilization in the alveolar cells.

In vitro experiments by Vassallo *et al.* (*ibid* 1973, **26,** 270) on the effects of NO_2 or nitrite ion (NO_2^-) on suspensions of rabbit alveolar macrophages indicate that both NO_2 and NO_2^- (10–15 mmoles) reduce the entry of *S. epidermidis* and *Pseudomonas aeruginosa* into macrophages and so limit the intracellular destruction of these organisms. Both agents increase glucose and pyruvate oxidation in resting macrophages. The data collected suggest the possibilities that NADPH and NADH may be oxidized by NO_2 or NO_2^- to $NADP^+$ and NAD^+, or that these agents may oxidize soluble thiol groups, including those of glutathione.

Acute toxicity

Hine *et al.* (*Toxic. appl. Pharmac.* 1970, **16,** 201) have assessed the acute toxicity of NO_2 in several species of experimental animal. Exposure times ranged from 5 to 1440 minutes

but, at concentrations up to 20 ppm. NO_2 caused no marked signs of irritation and had no behavioural effects. Microscopically, there was some doubtful evidence of congestion and interstitial inflammation for up to 48 hours after exposure. At 40 ppm, NO_2 produced eye irritation and an increase in respiration rate and volume. Occasionally, and especially in guinea-pigs, this concentration induced convulsions, which were attributed to laryngeal spasm. The critical toxic level for NO_2 was 50 ppm, lesser concentrations rarely producing lethal effects with exposures lasting less than 8 hours. The species examined, arranged in increasing order of sensitivity to NO_2, were guinea-pig, mouse and rat, rabbit and dog. Most deaths occurred within 2–8 hours, but 20% of the animals died later from pneumonitis and secondary bacterial infection. Respiration and heart rate increased, and terminal breathing was gasping and spasmodic. Motor activity was frequently reduced. In animals that died from the acute reaction, the lungs had a mottled surface, were fluid-filled and occupied several times their normal volume. Little alveolar damage was seen microscopically, but hyperaemia, oedema fluid and sometimes bronchial and bronchiolar necrosis were present. Interstitial fibrosis occurred in about one-third of survivors by day 30 and sometimes persisted for up to 6 months. Mortality from NO_2 exposure was increased by exercise or cold stress, adrenalectomy or high ambient carbon dioxide levels.

The need to look further into the long-term effects of exposure to low concentrations of NO_2 in the atmosphere has been stressed (*Cited in F.C.T.* 1972, **10,** 727). Much of the ambient NO_2 is derived from automotive exhaust, and in this regard, the findings of Stupfel *et al.* (*Archs envir. Hlth* 1973, **26,** 264) are cheering. Pathogen-free rats were exposed to automotive exhaust gases containing 50 ppm carbon monoxide plus 0·2 or 23 ppm nitrogen oxides (NO_x), together with carbon dioxide and aldehydes, for 8 hours daily, 5 days/week, for periods between 2·5 months and 2 years. Adverse effects were seen only with the exhaust containing 23 ppm NO_x; these included a decrease in body weight, a diminution in avoidance reflexes, and an increased incidence of spontaneous tumours in the ageing rats, a development tentatively attributed to the hydrocarbons in the exhaust gases. Survival, heart rate, electrocardiogram recordings and the incidence of renal and aortic lesions were not significantly affected by the exposure, even at the higher level.

[P. Cooper—BIBRA]

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TOXICOLOGY: ABSTRACTS AND COMMENTS

COLOURING MATTERS

2783. Sorting out methaemoglobinaemic agents

Lin, J.-K. & Wu, Y.-H. (1973). Studies on the mechanism of methemoglobin formation induced by aminoazo compounds. *Biochem. Pharmac.* **22,** 1883.

Aminoazo dyes are highly active in inducing methaemoglobinaemia in rats, an action that has been ascribed to their major reactive *N*-hydroxy metabolites. In an attempt to elucidate structure-activity requirements for methaemoglobin (MHb) production, rats were given an ip injection of one of several related compounds, dissolved in peanut oil, and blood was collected at intervals for MHb determinations. The compounds administered were 4-aminoazobenzene, *N*-methyl-4-aminoazobenzene, *N*,*N*-dimethyl-4-aminoazobenzene, *p*-aminophenol, *N*-phenylhydroxylamine, aniline, *p*-phenylenediamine, 4′-hydroxy-4-aminoazobenzene, *N*-acetyl-4′-hydroxy-4-aminoazobenzene and *N*-hydroxy-4-aminoazobenzene.

As an inducer of MHb, 4-aminoazobenzene proved to be more active than either its *N*-methyl or *N*,*N*-dimethyl derivative, but the effect of the two latter compounds was more persistent. The ring-hydroxylated metabolite was moderately active, but its *N*-acetyl derivative was less so. In comparative *in vitro* studies of MHb formation in isolated rat erythrocytes, the 3-hydroxy derivative was shown to be more active than the 4′-hydroxy form, but it is less likely to occur in significant amounts *in vivo*. Other possible intermediate metabolites of the dimethylaminoazobenzene—*N*-benzoyloxy-*N*-methyl-4-aminoazobenzene and *N*,*N*-dimethyl-4-aminoazobenzene-*N*-oxide—were also shown *in vitro* to be relatively active in inducing MHb formation.

These and other results established that the amino group was essential for MHb production, while the *p*-hydroxy group was not. *N*-Hydroxylation of the amino group appears to be necessary for the activation of 4-aminoazobenzene, but may account for only a fraction of the activation of aniline. Prior hydroxylation appears to be necessary to confer marked MHb-inducing ability *in vitro*, since 4-aminoazobenzene and its *N*-methyl and *N*,*N*-dimethyl derivatives all failed to produce MHb in isolated erythrocytes.

FLAVOURINGS, SOLVENTS AND SWEETENERS

2784. Teratogenicity study on coumarin

Grote, W. u. Weinmann, Inge (1973). Überprüfung der Wirkstoffe Cumarin und Rutin im teratologischen Versuch an Kaninchen. *Arzneimittel-Forsch.* **23,** 1319.

Coumarin derivatives have been widely used in anticoagulant therapy and rutin is used to counteract the increased capillary fragility that occurs in certain diseases. The dose of rutin given for this purpose is usually about 20 mg given three times daily (*Martindale: The Extra Pharmacopoeia*, edited by N. W. Blacow; 26th Ed., p. 1973; The Pharmaceutical Press, 1972).

Studies in pregnant mice (*Cited in F.C.T.* 1967, **5,** 723) and rats (Grote & Gunther, *Arzneimittel-Forsch.* 1971, **21,** 2016) yielded no evidence that coumarin administered orally either alone or combined with rutin had any teratogenic potential. Support for these negative findings has now been obtained in a third species treated by a different route.

White New Zealand rabbits were given a daily iv injection of coumarin or the coumarin–rutin combination "equivalent to 10 or 100 times the therapeutic dose" on days 6–18 of gestation. Control groups were given the dosage vehicle (10% 1,2-propylene glycol) or remained untreated. Body weight and food and water intake by the pregnant rabbits was unaffected by the treatment. The rabbits were killed on day 29 of gestation and the test and control groups showed no differences in the numbers of resorptions and dead foetuses or in foetal or placental weights. The incidence of malformations was not increased by any of the treatments, being 3·7 and 1·1% in the untreated and vehicle-treated controls, respectively, 4·0 and 4·6% in the groups given the lower doses of the two test materials and 1·1 and 1·6% in those given the higher doses. Earlier work in these animals had established a spontaneous malformation rate of 3·4%.

EMULSIFIERS AND STABILIZERS

2785. Delayed hypersensitivity reaction to carrageenan

Mizushima, Y. & Noda, M. (1973). Induction of delayed hypersensitivity by carrageenan. *Experientia* **29,** 605.

Carrageenan is known to interfere with various aspects of the immune response and, in this connexion, the importance of its cytotoxic effect on macrophages has been demonstrated (*Cited in F.C.T.* 1973, **11,** 1141). Carrageenan has also been shown to elicit a delayed hypersensitivity reaction in guinea-pigs (Mizushima & Noda, cited above).

Male guinea-pigs weighing 300–500 g were sensitized by intradermal injection of 0·025 1·5 mg carrageenan suspended in saline. Some 2–3 wk later, 0·05 ml of a 0·5–0·05% carrageenan suspension was injected into the skin on the opposite side of the back. A weak skin reaction developed 5 hr after this challenge injection both in the sensitized animals and, to the same degree, in non-sensitized controls. The following day, however, the sensitized animals showed an intensive erythema frequently associated with a pale central area, bleeding and induration and persisting for more than 72 hr. Granulomas consisting mainly of lymphoid cells and scattered giant cells were demonstrated at 48 and 72 hr, and systemic passive transfer studies using peritoneal exudate cells from carrageenan-sensitized guinea-pigs showed the reaction to be a typical delayed-type hypersensitivity.

PRESERVATIVES

2786. Effect of diet on nitrite-induced methaemoglobinaemia

Stoewsand, G. S., Anderson, J. L. & Lee, C. Y. (1973). Nitrite-induced methaemoglobinemia in guinea pigs: Influence of diets containing beets with varying amounts of nitrate, and the effect of ascorbic acid, and methionine. *J. Nutr.* **103,** 419.

Nitrites have come under a cloud recently because of their ability to produce nitrosamines by reaction with secondary amines (*Cited in F.C.T.* **12,** 156). A longer-established hazard is the methaemoglobinaemia induced by high levels of nitrites, which may be formed from nitrates by bacterial action during food storage or in the alimentary tract (*ibid* 1967, **5,** 425). Vitamin A supplementation may alleviate the toxic effects of nitrites (*ibid* 1969, **7,** 543) and some protection against methaemoglobinaemia may also be afforded by methionine (Mortensen, *Archs Biochem. Biophys.* 1953, **46,** 241) and ascorbic acid (Kociba & Sleight, *Toxic. appl. Pharmac.* 1970, **16,** 424), although some workers have reported negative results with the latter compound (Bolyai *et al. ibid* 1972, **21,** 176; Kilgore *et al. Am. J. clin. Nutr.* 1964, **14,** 52).

Stoewsand *et al.* (cited above) maintained young, male guinea-pigs on beet-supplemented diets containing either 242 or 1998 ppm nitrate-nitrogen, the higher level being the result of application of an ammonium nitrate fertilizer to the growing beets. Other guinea-pigs were fed a purified diet containing 148 or 1798 ppm nitrate-nitrogen. The nitrite-nitrogen content of all four diets was in the 1·7–4·0 ppm range. After 2·5–4 wk, the blood levels of methaemoglobin in the four groups did not differ significantly, all falling in the range of 1·0–1·8% of the total haemoglobin. However, methaemoglobin levels were significantly increased by the oral administration of 7·6 or 15·0 mg nitrite/kg body weight. The effect of the lower nitrite dose was more severe in animals receiving a high-nitrate diet; and with the higher dose of nitrite animals on the low-nitrate beet diet were much less severely affected than the other three groups (having a mean methaemoglobin level of 27% compared with 44–46%). With a nitrite dose as high as 22·8 mg/kg, the methaemoglobin levels of all four groups were approximately 60%, but mortality was lower in the group fed the low-nitrate beet diet than in the other groups. These findings suggested that nitrate could greatly exacerbate the effects of nitrite and that some factor present in the beet diet could give some degree of protection against nitrite toxicity.

In a second experiment, guinea-pigs were fed an isolated soya-bean protein diet supplemented with 0, 0·02 or 1·0% ascorbic acid and/or 0 or 1·0% methionine. The methaemoglobinaemia induced by a dose of 7·6 mg nitrite/kg was less severe in the animals given ascorbic acid at the 1·0% than at the 0·02% level and an additional 50% reduction was obtained when the diet contained 1% levels of both ascorbic acid and methionine. Increasing the level of each additive to 2%, however, brought about no further reduction in the nitrite-induced methaemoglobinaemia.

AGRICULTURAL CHEMICALS

2787. Ironing out carbaryl discrepancies

Weil, C. S., Woodside, M. D., Bernard, J. B., Condra, N. I., King, J. M. & Carpenter, C. P. (1973). Comparative effect of carbaryl on rat reproduction and guinea-pig teratology when fed either in the diet or by stomach intubation. *Toxic. appl. Pharmac.* **26,** 621.

Recent publications have brought to light the relative rarity of studies comparing the effects resulting from dietary and gavage administration of a given test material. One such study has been carried out on carbaryl (*N*-methyl-1-naphthylcarbamate) by the authors named above. Discrepancies have been noted in the results of different studies on the effect of this insecticide on animal reproduction and in assessments of its teratogenic potential

(*Cited in F.C.T.* 1973, **11,** 331), and this paper does something to underline the importance of the route of administration as a factor in this apparent lack of agreement.

In concurrent three-generation studies, the effects of feeding carbaryl to rats at dietary levels providing daily doses up to 200 mg/kg/day were compared with the effects of its oral intubation in doses up to 100 mg/kg/day. In addition, the authors describe a teratological study in which pregnant guinea-pigs were given up to 300 mg/kg/day in the diet or up to 200 mg/kg/day by oral intubation on selected days, varying in total from 1 to 15, between days 10 and 24 of gestation.

No teratological effects were observed in any trial with either rats or guinea-pigs. In rats, the only significant effects following the feeding of 200 mg carbaryl/kg/day in the diet were an inhibition of weight gain before the first mating period and an increase in the gestation period for the F_{2a} generation. In the same species, however, intubation of 100 mg carbaryl/kg/day inhibited cholinesterase, reduced weight gain, increased the early mortality rate, lengthened gestation and reduced both the numbers and viability of offspring in the F_2 and F_3 generations. In guinea-pigs, intubation with 200 mg carbaryl/kg/day increased the early mortality rate and reduced weight gains, but these effects were not seen after dietary administration.

These results indicate that the mode of administration of carbaryl may have considerable influence on its toxic effects, and support the call for the more careful design of experiments.

2788. Experimental carcinogenesis from fumigants

Olson, W. A., Habermann, R. T., Weisburger, Elizabeth K., Ward, J. M. & Weisburger, J. H. (1973). Induction of stomach cancer in rats and mice by halogenated aliphatic fumigants. *J. natn. Cancer Inst.* **51,** 1993.

Ethylene dibromide (EDB) is known to have hepatotoxic potential, and in rats has been shown to induce a rise in liver triglyceride concentration within 12 hr (*Cited in F.C.T.* 1973, **11,** 1150). In the paper cited above a more serious hazard of EDB and of 1,2-dibromo-3-chloropropane (DBCP) is described.

EDB and DBCP are used as a grain pesticide and soil fumigant respectively. Tolerances for residues of these compounds on treated grains and vegetables are based on their inorganic bromine residues. Although their volatility makes it unlikely that toxic amounts of EDB or DBCP will be retained by processed or cooked foods, exposure of agricultural and store workers is a more likely possibility. A threshold limit of 200 ppm or 145 mg/m^3 has been proposed for EDB in working atmospheres, but none has been set for DBCP.

Rats and mice were given intragastric doses of one of the compounds 5 times/wk, groups of 50 animals of each sex being used for each dose level. DBCP was given to male and female rats initially in doses of 12 or 24 mg/kg/day, raised after 14 wk to 15 or 30 mg/kg/day. The male mice received 80 or 160 mg/kg/day and the females 60 or 120 mg/kg/day initially, but these levels were raised after 14 wk to 100 or 200 mg/kg/day and after 28 wk to 130 or 260 mg/kg/day for both sexes. The earliest observed effect of DBCP, at 14 wk, was the appearance of mammary tumours in several female rats. These tumours progressed, becoming larger and sometimes ulcerated, and after a few weeks the animals had to be killed. Five males killed after 40 wk showed squamous-cell carcinomas of the stomach, and more had developed similar carcinomas by wk 50. This type of tumour also appeared in a high percentage of mice of both sexes.

The EDB dose levels were initially 40 and 80 mg/kg/day for rats, but dosing at the top

level was discontinued between wk 16 and 30, after which treatment was resumed with doses of 40 mg/kg/day. The mice were first given 60 or 120 mg EDB/kg/day, but after wk 42 both groups received 60 mg/kg/day. EDB initially depressed weight gain in rats, and within 10 wk of the start of treatment one male and one female developed squamous-cell carcinomas of the stomach. This tumour was eventually present in 80% of all the males. There was a lower tumour incidence in male rats on the higher dose of EDB than in those on the lower (62 and 98%, respectively). This may have been due to earlier mortality in the former group or to the fact that the top-level dosing was interrupted for 14 wk because of overt toxicity. The tumour incidence in females was lower than in males, but was still appreciable. The tumours originated in the forestomach, invaded the local tissues, and eventually metastasized throughout the abdominal cavity. Preliminary results obtained in mice, which were started on the EDB treatment at a later stage of the study, indicated that squamous-cell carcinomas were also developing in this species.

These results suggest that occupational exposure to either DBCP or EDB may constitute a health hazard, and care should be taken to avoid inhalation of either material or absorption via some other route, at least until further data have been acquired.

2789. More about paraquat lung lesions

Kimbrough, R. D. & Linder, R. E. (1973). The ultrastructure of the paraquat lung lesion in the rat. *Envir. Res.* **6,** 265.

Previous work published by this group (*Cited in F.C.T.* 1972, **10,** 702) has demonstrated oedema, epithelial swelling, increased collagen and alterations in the ribosomes of the membranous pneumocytes in the lungs of animals after a single dose of paraquat.

The paper cited above describes lung lesions produced by feeding paraquat to female Sherman rats at a level of 500 ppm in their diet for 1–11 wk. The lungs of rats killed 1 or 3 wk after the beginning of treatment appeared to be normal, as were those of the control rats. Of the four animals examined after 5 wk, two had normal lungs, one showed grossly visible areas of lung consolidation and one showed ultrastructural changes. The areas of consolidation contained a proliferation of epithelial cells surrounded by inflammatory cells and fibroblasts. The alveoli of this animal, and of others examined later, contained prominent amorphous material staining faintly pink with eosin but containing no lipid. By electron microscopy the amorphous material was seen to be highly osmiophilic and arranged in concentric whorls adjacent to or surrounded by a less electron-dense lattice-like material. Oedematous and vacuolated membranous pneumocytes and endothelial cells were observed. After 7 wk, all exposed rats showed these ultrastructural changes in the alveoli, and after 11 wk, three of the four rats killed were similarly affected.

The membranous (type 1) pneumocytes, the type affected by paraquat, are instrumental in the removal of pulmonary surfactant (Kikkawa & Suzuki, *Lab. Invest.* 1972, **26,** 441), the accumulation of which is known to be abnormal in paraquat-affected lungs (*Cited in F.C.T.* 1974, **12,** 781).

PROCESSING AND PACKAGING CONTAMINANTS

2790. Respiratory problems from PVC pyrolysis products

Sokol, W. N., Aelony, Y. & Beall, G. N. (1973). Meat-wrapper's asthma. A new syndrome? *J. Am. med. Ass.* **226,** 639.

Stevens, J. J. (1974). Meat-wrapper's asthma. *J. Am. med. Ass.* **227,** 1005.
Jaeger, R. J. & Hites, R. A. (1974). Pyrolytic evaporation of a plasticizer from polyvinyl chloride meat wrapping film. *Bull. env. contam. & Toxicol. (U.S.)* **11,** 45.

The first paper cited above describes three cases of respiratory distress, including breathlessness, wheezing and coughing, experienced by workers employed as meat wrappers. This syndrome apparently followed exposure to fumes produced during the cutting of PVC film with a hot wire. There were no personal or family histories of eczema, hay fever or asthma, and skin tests for common allergens were negative in all cases. Blood tests and chest X-rays were normal but there was some impairment of pulmonary function, which improved with bronchodilation. Symptoms developed after periods of exposure varying from 20 min to 5 hr and disappeared when use of the hot wire ceased. Two more cases of workers who experienced similar symptoms under comparable conditions are described by Stevens (cited above). In these cases, however, a reaction to either grass or dust antigens was recorded. All sufferers from this syndrome were cigarette smokers.

Similar complaints from other workers involved in the manufacture or use of PVC food-wrapping materials led the Bureau of Occupational Safety and Health in the USA to investigate the pyrolytic products of PVC film, and it appears that an unpublished preliminary communication on this work has identified chlorobutane, benzene, toluene, 1-chloro-2-ethylhexane, 2-ethyl-1-hexanol, benzyl chloride and hydrogen chloride as products of the pyrolysis of PVC film at about 400 F. This information is given in the third paper cited above, the authors of which also describe the case of a worker who, in addition to suffering from complaints similar to those already described, noted an oily material in the vicinity of the meat-wrapping machinery. In subsequent laboratory investigations, samples of the PVC film were heated for 1·5 hr at 275–350°F. The oily condensate obtained was extracted with acetone and subjected to gas chromatography and mass spectrometry, which indicated that the material in question was dicthylhexyl adipate, the compound originally employed in the PVC film as a plasticizer.

2791. More on phthalate metabolism

Albro, P. W. & Thomas, R. O. (1973). Enzymatic hydrolysis of di-(2-ethylhexyl) phthalate by lipases. *Biochim. biophys. Acta* **360,** 380.

Di-(2-ethylhexyl) phthalate (DEHP) has been reported in the tissues of patients transfused with blood that had been stored in PVC packs (*Cited in F.C.T.* 1971, **9,** 910; *ibid* 1973, **11,** 914), but there has been some controversy over whether such findings are due to accidental contamination during the tissue-extraction procedures involved in the analyses. Work in rats has indicated that low doses of DEHP given iv are rapidly metabolized and excreted within 24 hr, although larger doses may be retained for much longer periods (Schulz & Rubin, Phthalic Acid Esters Conference, NIEHS, Pinehurst, N.C., 6–7 September 1972). After oral administration of labelled DEHP, a significant percentage of tissue radioactivity was at no time associated with the intact diester (Schulz & Rubin, *Envir. Hlth Perspec.* 1973, **3,** 123). DEHP given orally has been shown to be degraded by the contents of the rat caecum or small intestine to a single metabolite, tentatively identified as mono-(2-ethylhexyl) phthalate (MEHP) (Rowland, *Fd Cosmet. Toxicol.* 1974, **12,** 293). The presence of five other metabolites in rat urine has suggested that this initial hydrolysis is followed by ω-oxidation and (ω-1)-oxidation of the remaining ester group, probably in the liver (*Cited in F.C.T.* 1973, **11,** 915).

Further information on possible sites of hydrolysis has been obtained from the present

in vitro study, in which DEHP was incubated with 15 tissue lipases and several heparin-activated lipoprotein lipases, chiefly derived from the rat. Hydrolysis to MEHP was found to occur in all preparations except those containing glycerol-ester hydrolase (pancreatic lipase; EC 3.1.1.3) and sterol-ester hydrolase (cholesterol esterase; EC 3.1.1.13). Rat pancreatic hydrolase was able to convert DEHP completely to MEHP, and high activity was also demonstrated by homogenates of rat liver and intestinal mucosa, while preparations of rat kidney, lung, plasma lipoprotein and adipose tissue showed lower degrees of potency. A comparison of intestinal homogenates from rats, mice, hamsters and guinea-pigs revealed no great differences between species, although there were considerable variations between different mouse strains. No difference was found between young and old rats, but male rats had a higher activity than females. Of all the tissues examined, only the alkaline lipase of rat liver was able to hydrolyse MEHP further to phthalic acid, at 2% of the rate at which it hydrolysed DEHP.

The findings suggested that while dietary DEHP would have little opportunity to be absorbed intact, injected DEHP would probably survive long enough for all the tissue lipases and lipoprotein lipases tested to play some part in its hydrolysis.

2792. Phthalates and the aquatic life

Williams, D. T. (1973). Dibutyl- and di-(2-ethylhexyl)phthalate in fish. *J. agric. Fd Chem.* **21,** 1128.

Sanders, H. O., Mayer, F. L., Jr. & Walsh, D. F. (1973). Toxicity, residue dynamics, and reproductive effects of phthalate esters in aquatic invertebrates. *Envir. Res.* **6,** 84.

Phthalic acid esters, of which the most widely used are di-2-ethylhexyl phthalate (DEHP) and di-*n*-butyl phthalate (DBP), have been identified as contaminants of certain inland and coastal waters in North America, particularly in areas contiguous to industrial centres.

To put the problem as it may exist in Canada into perspective, Williams (cited above) has made a preliminary survey of the levels of these two major phthalates in fish available to the Canadian consumer. The highest concentrations recorded were 160 ppb ($b = 10^9$) for DEHP and 78 ppb for DBP found in a sample of processed canned tuna fish. These esters were not, however, detected in more than trace amounts in the varieties of processed frozen fish examined (trout, ocean perch, mackerel, sole, oyster and scallop) or in unprocessed catfish or pickerel. A relatively high level of DEHP (104 ppb) was found in a sample of unprocessed eel, and lower levels, sometimes with DBP, were found in salmon but not in sardine, crab, shrimp or baby clams.

The number of samples studied in this work was small but the findings are not in conflict with a recent report (Stalling *et al. Envir. Hlth Perspec.* 1973, **1** (3), 159) that DEHP and DBP are metabolized by fish, since this indicates that high residue levels would only be expected in organisms continuously exposed to phthalate esters. The highest phthalate levels have, in fact, been recorded in fish from waters near industrial areas and in hatchery fish fed diets contaminated with these esters. In an extension of previous observations, Sanders *et al.* (second paper cited above) have demonstrated that invertebrates (several species of crustacea and insects) exposed to ^{14}C-labelled DEHP and DBP exhibit a rapid uptake initially, so that within 2 wk body residues ranged from 70 to 13,600 times that of the water concentration. All but about 6% of these residues were lost again after the organisms had been in phthalate-free fresh water for a period of 10 days.

These workers have also pioneered studies on the biological significance of these phtha-

late residues, at least in the scud (*Gammarus pseudolimnaeus*). The toxicity of DBP was low but increased with duration of exposure, the median tolerance limit (TL_{50}) being 7 ppm at 24 hr and 2·1 ppm at 96 hr. DEHP was relatively innocuous to scud, with a 96-hr TL_{50} of > 32 ppm. The 96-hr TL_{50} of DBP to crayfish was > 10 ppm. When waterfleas (*Daphnia magna*) were exposed continuously for a complete life cycle (21 days) to 3–30 ppb DEHP, the numbers of offspring were reduced by 60–83%. Overall. these findings are considered to give little cause for concern in relation to the aquatic organisms themselves, provided exposure is not continuous, but in view of the importance of such invertebrates in the food chain, their ability to accumulate phthalates may conceivably affect the growth and reproduction of a wide variety of predatory vertebrates.

THE CHEMICAL ENVIRONMENT

2793. Bone of contention for fluoride

Chan, M. M., Rucker, R. B., Zeman, F. & Riggins, R. S. (1973). Effect of fluoride on bone formation and strength in Japanese quail. *J. Nutr.* **103,** 1431.

While the effects of inorganic fluoride in blocking the resorption of newly deposited bone mineral (*Cited in F.C.T.* 1971, **9,** 278) and in increasing bone density have been described in several animal species, the relationship between these phenomena has received relatively little attention. The experiments reported here, involving the administration of various dietary levels of calcium and fluoride to an avian species with a high tolerance to the halogen, were designed to probe fluoride-induced changes in bone metabolism.

Immediately after hatching, groups of Japanese quail were fed diets containing 1·2 or 0·4% calcium, in each case with and without 0·075% fluoride. After 11 days of treatment, the lower level of calcium in the fluoride-free diet produced a 23% reduction in body weight, a 38% decrease in bone ash and a twofold increase in bone pyrophosphatase levels, compared with control birds fed 1·2% dietary calcium. Addition of fluoride to the low-calcium diet enhanced the calcium retention (as measured by ip injection of ^{45}Ca), growth rate and bone ash. The bone calcium/phosphorus ratio was not significantly disturbed by the experimental diets, but bone magnesium rose above control values in both of the fluoride-supplemented groups and in the group on the low-calcium diet.

In a second experiment, all birds were fed the control diet (1·2% calcium) for 10 days, after which they were fed one of the other diets for 35 days. In this test, quail fed the low-calcium diet showed none of the signs of marked calcium deficiency seen in the previous experiment. In the fluoride-supplemented group, significant ($P < 0·05$) increases in periosteal bone formation were detected, following injection of the birds with tetracycline to facilitate histological examination, but the absence of any increase in cortical thickness indicated that the bone formation was being balanced by resorption of endosteal bone. Use of the Von Kossa staining technique showed that mineralization of these new bone layers was normal, but there appeared to be some increase in the number of osteons in bone sections from fluoride-treated quail. An increase in the number of osteons in a given area tends to reduce both the tensile strength and the elastic modulus of bone, and there was, in fact, a reduction of approximately 30% in the torsional strength of the bone in fluoride-supplemented quail, compared with that in control birds.

The authors conclude that while dietary fluoride supplementation encourages calcium and magnesium retention in bone, this accelerated mineralization appears to be accompanied by a decrease in bone strength, at least in the quail.

2794. Iron in the cell

Ganote, C. E. & Nahara, G. (1973). Acute ferrous sulfate hepatotoxicity in rats. An electron microscopic and biochemical study. *Lab. Invest.* **28,** 426.

For many years, inorganic iron salts have been used in the oral treatment of iron-deficiency anaemia, but chronic systemic intoxication from such therapy is very rare. Nevertheless ingestion of excessive quantities of iron preparations, of which ferrous sulphate is probably the most widely used, have from time to time produced acute toxic effects, including gastro-intestinal irritation, cardiovascular collapse and hepatic necrosis, and these reactions have occasionally proved fatal. In order to investigate the aetiology of iron-induced liver injury, the authors cited above examined the ultrastructural pathology of the condition in rats treated with ferrous sulphate, using recently improved techniques of liver fixation.

Administration of ferrous sulphate to rats by gavage in a dose of 250 or 500 mg iron/kg body weight induced peripheral vasoconstriction, moderate falls in blood pressure and listlessness. With the lower dose, few animals developed necrosis, and in most cases electron microscopy revealed only irregular dense granules in the intracristal lumen of the parenchymal mitochondria. These were found at all intervals between 1 and 48 hr after dosing. At the higher dose level, there was a 70% mortality rate within 12 hr. Marked swelling of scattered midzonal and periportal liver cells developed within 1 hr and areas of necrotic cells associated with an insurgence of acute inflammatory cells were visible under the light microscope within 3 hr. Active cellular swelling and necrosis continued for up to 12 hr, after which fatty infiltration increased and the Kupffer cells were found to contain large, intensely iron-positive inclusions. The earliest changes observed under the electron microscope in parenchymal cells irreversibly injured by iron occurred in the mitochondria. These were either swollen or condensed, and both forms developed aggregates of particulate material in the matrix, which in the swollen ones also showed amorphous densities thought to be composed of denatured proteins. In mitochondria isolated from the livers of the rats given the iron preparation, dense granules were present in the intracristal lumen. These granules were absent from the liver mitochondria of control animals, and so were postulated to occur only in cells actively adsorbing excessive iron loads.

Polarographic studies on mitochondria isolated from animals with damaged livers showed a loss of respiratory control between 3 and 5 hr after iron administration. Altered mitochondrial function was demonstrable only in livers showing evidence of cell damage and the timing of these events suggested a possible causal relationship between them, although this could not be confirmed. To investigate the theory that the mitochondrial effects might be secondary to liver anoxia, factors known to improve mitochondrial respiratory capacity after experimental shock were added to the isolated organelles. The addition of magnesium, albumin or a cofactor mixture failed to enhance mitochondrial function after iron injury. It was therefore concluded that iron hepatotoxicity may be the result of primary mitochondrial damage.

2795. Lead encephalopathy in rat and man

Clasen, R. A., Hartmann, J. F., Starr, A. J., Coogan, P. S., Pandolfi, Sylvia, Laing, Iris, Becker, Ruth & Hass, G. M. (1974). Electron microscopic and chemical studies of the vascular changes and edema of lead encephalopathy. A comparative study of the human and experimental disease. *Am. J. Path.* **74**, 215.

Lead encephalopathy is readily induced in suckling rats by administration of water-soluble lead salts to the mother (*Cited in F.C.T.* 1969, **7**, 260). The extent to which this lesion resembles the one induced in man is not known with certainty and the present study was conducted in an effort to bridge this gap.

When female Sprague-Dawley rats were placed on a synthetic diet containing 4% lead subacetate about 1 wk after delivery, the suckling animals failed to develop normally and after 3 wk many became paralysed and died. Survivors were killed 24–40 days after birth along with paired controls of the same age. In the animals most severely affected and showing hind limb paralysis, the white matter of both the cerebellum and cerebrum contained a protein-rich fluid separating the myelinated nerve fibres. This stained positively with the periodic acid–Schiff (PAS) technique and was associated with glial cells, the cytoplasm of which stained intensely. A similar type of fluid and vacuolation were seen in focal areas of the basal ganglia and cerebellum, in which areas there were also PAS-positive globules surrounding blood vessels. Ultrastructurally this material consisted of strongly osmiophilic cytoplasmic inclusions which were not membrane-limited and were located in the perivascular astrocytes. Intervascular strands consisting of a basement membrane surrounding endothelial cytoplasm were also found.

Similar pathological changes were observed in brain tissue taken at autopsy from cases of lead encephalopathy examined by the authors. In this human material, derived from nine young children and two adults, the PAS-positive globules were predominantly but not (as in the rat) exclusively within the perivascular astrocytes, some being found within the vessel wall in the human tissue.

Chemically, experimental lead encephalopathy with morphologically demonstrable oedema was characterized by an increased brain content of water, sodium and serum albumin compared with untreated controls. In comparison with the serum levels, the increase in water was disproportionately greater than that of sodium or albumin and there were no demonstrable changes in chloride or potassium levels.

2796. An immunological hypothesis for talc reactions

Carson, S. & Kaltenbach, J. P. (1973). Murine immunoglobulin response to sterile talc injection. *Expl mol. Path.* **18**, 18.

Talc (a natural magnesium silicate) is known to cause proliferative inflammation when embedded in tissues, particularly of the bronchi and bronchioli (*Cited in F.C.T.* 1971, **9**, 594). Observations that the injection of talc increased the γ-globulin fraction of serum proteins led to an investigation of the possible immunological basis of the local talc reaction.

Mice were given sc either a single injection of 300 or 600 mg talc, or two contralateral injections each of 300 mg, or two separate injections of 300 mg separated by an interval of 24, 48 or 72 hr. The animals were killed after intervals of 12 and 24 hr after the final injection and then at 24-hr intervals up to 144 or 216 hr (the latter after the contralateral injections), and blood samples were taken for immunoglobulin determinations. Paired controls were given sterile saline injections of comparable volume. Care was taken throughout to use sterile techniques.

Injected talc induced a rise in serum IgM at 24 hr and a rise in serum IgG at 24–48 hr. This effect was independent of the size of the dose and the number of injections. There was no significant difference between the immunoglobulin levels following single injections of 300 or 600 mg. or two injections of 300 mg. although second injections sometimes resulted in a second transient rise in IgM. Serum levels of IgG after a second injection varied with the interval between successive injections. Histological examination of the injection site showed a heavy polymorphonuclear-cell infiltration at 24 hr, perivascular mononuclear-cell infiltration at 48 and 72 hr and fibroblastic proliferation at 96 hr.

The tentative hypothesis put forward to explain these results is that talc produces local cellular necrosis, releasing lysosomal proteolytic enzymes which modify exposed cellular or extracellular proteins sufficiently for them to act as foreign proteins. These modified proteins are then adsorbed on to talc particles and stimulate the host's immune system. The authors suggest this as a working hypothesis for further studies on the sequence of events involved and the benefit of the response to the host.

2797. Getting rid of inhaled acetone

DiVincenzo, G. D., Yanno, F. J. & Astill, B. D. (1973). Exposure of man and dog to low concentrations of acetone vapor. *Am. ind. Hyg. Ass. J.* **34,** 329.

The absorption and excretion of acetone following brief exposure to high concentrations has been described fairly recently (Raleigh & McGee, *J. occup. Med.* 1972, **14,** 607), but its fate in subjects exposed to concentrations below the threshold limit value of 1000 ppm has until now received relatively little attention.

In the study cited above, male volunteers were exposed for 2 or 4 hr to acetone concentrations of 100 or 500 ppm, and dogs were exposed for 2 hr to concentrations of 100, 500 or 1000 ppm. In the men, no untoward effects or subjective symptoms were observed, and clinical chemistry and haematology were unaffected. The concentrations of acetone in the breath and blood of both species were directly proportional to the concentrations inhaled, although no such correlation was found with levels in human urine. In man, some 75–80% of the inspired vapour was absorbed into the blood, the other 20–25% remaining in the dead space volume. The proportion absorbed was greater than that in the dog, a finding attributed to the dog's slightly lower minute volume. Disappearance from the blood was independent of concentration in both species and the half-life of acetone in the blood was approximately 3 hr. Breath concentrations of less than 1 and 5 ppm were found 7 hr after exposure of the volunteers for 2 hr to 100 or 500 ppm, respectively, but the shape of the elimination curves suggested that repeated daily exposures to higher concentrations could result in some accumulation. Spasmodic light exercise (jogging) approximately doubled acetone concentrations in the breath both during and after exposure, but increasing the exposure time from 2 to 4 hr produced a less than twofold increase in post-exposure elimination from the lungs, a reflection of the loss of acetone by other routes.

In view of these findings, the authors suggest that dogs would be a suitable species for the evaluation of human exposure to acetone in high concentrations or under diverse experimental conditions, provided the metabolic and physiological differences between the two species were taken into account.

2798. The toxicity of branched-chain alcohols

Scala, R. A. & Burtis, E. G. (1973). Acute toxicity of a homologous series of branched-chain primary alcohols. *Am. ind. Hyg. Ass. J.* **34,** 493.

The branched-chain primary aliphatic alcohols are widely used in industry as solvents and lubricants and as starting materials for various other compounds, such as ester plasticizers. However, although individual members of the class have featured in certain range-finding studies (*Cited in F.C.T.* 1970, **8,** 467) and, at the lower end of the scale, a subacute toxicity study on isoamyl alcohol has recently been published (Carpanini *et al. Fd Cosmet. Toxicol.* 1973, **11,** 713), most toxicological investigations have centred on their straight-chain relatives (*Cited in F.C.T.* 1967, **5,** 729). In the present study, acute oral, dermal, eye and inhalation tests were conducted on a homologous series of branched-chain alcohols containing 5, 6, 8 (both the isooctyl and the 2-ethylhexyl forms), 9, 10, 13 or 16 carbon atoms.

Oral LD_{50} values in rats provided evidence of a fairly low acute toxicity and showed no consistent increase with increasing molecular weight, ranging from 1·48 g/kg for isooctanol to 4·75 g/kg for tridecanol. Hexadecanol produced no deaths when given in an oral dose of over 8 g/kg. Toxic signs, characteristic of primary alcohols, were central nervous system (CNS) depression and laboured respiration, and gastro-intestinal irritation was seen with the amyl, hexyl and 2-ethylhexyl alcohols. When dose levels of 2·6 or 3·2 g/kg were applied to rabbit skin in undiluted form under occlusive binding for 24 hr, there was evidence in all cases of percutaneous absorption, but CNS depression, indicative of percutaneous toxicity, was seen only with amyl, hexyl and isooctyl alcohols, and there was only one death, in an animal treated with 2·6 g isooctanol/kg. The degree of skin irritation decreased from severe to slight with increasing molecular weight. Instillation into the conjunctival sac of the rabbit produced severe irritation, except in the cases of tridecanol and hexadecanol, which were only moderate and slight, respectively, in their effect. When mice, rats and guinea-pigs were exposed for 6 hr to aerosols of amyl alcohol (14 ppm) or isononanol (21·7 ppm), mucosal irritation and CNS depression were seen and there were some fatalities, with signs of kidney and lung damage from amyl alcohol. Exposure for a similar period to almost saturated atmospheres of the other alcohols produced mild to moderate mucosal irritation, which varied inversely with the molecular weight, but CNS depression was clearly produced only by 2-ethylhexanol, and there were no deaths in these groups.

2799. Toxicity of alkylformamides

Pham-Huu-Chanh, Azum-Gélade, M. C., Nguyen-van-Bac & Nguyen-Dat-Xuong (1973). Toxicological studies of the *N*-*n*-propyl and *N*-*n*-butyl derivatives of formamide. *Toxic. appl. Pharmac.* **26,** 596.

The group responsible for a project designed to establish the toxicity and pharmacological properties of derivatives of formamide, acetamide and propionamide has added some data on formamide derivatives to the information on alkylacetamides already published (*Cited in F.C.T.* 1971, **9,** 749).

Immediate and delayed acute toxicity tests were performed in rats and mice by ip injection of formamide, *N*-*n*-propylformamide (PF), *N*,*N*-di-*n*-propylformamide (DPF), *N*-*n*-butylformamide (BF) and *N*,*N*-di-*n*-butylformamide (DBF). The LD_{50} of formamide was 5·7 g/kg in the rat and 4·6 g/kg in the mouse, compared with 0·44 and 0·38 g/kg, respectively, in the case of PF. Values of the same order were established for the other compounds, with a general trend towards a slight increase in toxicity with further increases in carbon-chain length and in molecular weight. The acute crisis time (at which the mortality rate stabilized) varied widely, from 15–17 days for PF to 18–24 hr for DBF. Rats

proved to be consistently more resistant to formamide and its derivatives than mice. Near-lethal doses in mice produced early central nervous excitation, cycles of excitatory and tonic crises, and then depression and hypothermia. Higher doses produced not excitatory crises but paralysis. DBF was exceptional in that high doses were convulsant in mice. In rats, PF and BF produced almost continuous depression.

Doses of 0·5–0·65 g PF/kg produced liver changes, which included cytoplasmic microvacuolization. Doses of 1·4–2·34 g PF/kg caused vascular congestion of the heart, spleen, kidneys, adrenals, liver, lungs, pancreas, thyroid, testes and abdominal aorta in mice that survived for 1 month, these changes being particularly marked in the liver and spleen. Incipient toxic changes in the proximal kidney tubules and interstitial oedema of the pancrease were also noted. DPF produced none of these effects, but congestive changes appeared in mice given a lethal dose of BF, and the survivors from this group showed enlarged spleens with red-pulp hyperplasia, an effect also seen to a more marked degree with DBF. Rats given a lethal dose of PF showed foci of necrosis on the liver surface and hepatitis, those given a lethal dose of DPF showed intense congestion of the small intestine, and BF and DBF caused a severe pancreatitis. Survivors showed no detectable cellular damage 1 month after administration of any of these alkylformamides.

Male rats given daily ip doses equivalent to 5% of a sublethal dose 5 times weekly for a total of 36 doses showed no behavioural changes or cellular lesions. These doses (5, 9·5, 2·5 and 12 mg/kg/day for PF, DPF, BF and DBF, respectively) modified the growth pattern. PF and DPF causing some increase in growth rate and the others a slight reduction. All four compounds increased the total blood proteins, particularly the albumin fraction in relation to the α-globulin fraction, and reduced serum potassium levels after 15 injections but not after 36 except in the case of BF. Urinary sodium, potassium and calcium were found to be depressed after 15 injections but elevated after 36.

2800. Azoxyethane and the foetus

Griesbach, U. (1973). Selektive Erzeugung von Missbildungen durch Azoxyäthan während der Frühentwicklung der Ratte. *Naturwissenschaften* **60,** 555.

The azoxyalkanes are of interest because of the involvement of the methane derivative in cycad carcinogenesis and because of their chemical relationship to derivatives of the alkylnitrosamines and alkylhydrazines (*Cited in F.C.T.* 1966, **4,** 192).

Azoxyethane administered to BD rats in a single injection during the second half of gestation has been shown to result in the development of malignant tumours of the nervous system in the offspring. In the work cited above, rats of an inbred BD strain were given a single sc dose of 30 or 50 mg azoxyethane/kg between days 3 and 12 of gestation and the offspring were observed throughout life.

Azoxyethane administration was particularly associated with abnormalities of the eye (notably microphthalmia, anophthalmia, and hypoplasia and aplasia of the optic nerves) and, in the case of the higher dose level, also of the brain (hydrocephalus internus). In 145 offspring of the rats treated between days 3 and 8·5 after mating, neither malformations nor tumours were found, while treatment between days 8·5 and 10 resulted in 65 eye malformations and 18 cases of hydrocephalus but no tumours among the 115 offspring. In contrast, treatment between days 10 and 12 caused no malformations of either the eyes or brain of the young, but tumours of the central and peripheral nervous systems occurred.

Azoxyethane thus exerts either a teratogenic or carcinogenic effect on the developing rat foetus, depending on the stage of development reached at the time of exposure. Azoxymethane, on the other hand, has been shown to be neither teratogenic nor carcinogenic in these circumstances.

2801. Effects of bis-chloromethyl ether

Thiess, A. M., Hey, W. u. Zeller, H. (1973). Zur Toxikologie von Dichlordimethyläther —Verdacht auf kanzerogene Wirkung auch beim Menschen. *Zentbl. ArbMed. ArbSchutz* **23**, 97.

The long-term carcinogenic potential of bis-chloromethyl ether (BCME) in animals and man (*Cited in F.C.T.* 1974, **12**, 551) has recently caused some disquiet. Official recognition of this concern has resulted in a statement by HM Chief Inspector of Factories that "the carcinogenic risk of these compounds is such that no exposure or contact by any route—respiratory, skin or oral, as detected by the most sensitive methods should be permitted" (*Annual Report* 1972 *of HM Chief Inspector of Factories*. Department of Employment. HMSO. London, 1972; *Cited in F.C.T.* 1974, **12**, 541). The US Occupational Safety and Health Administration has now confirmed its emergency standards for the handling of certain carcinogens, which include BCME (*Federal Register* **39**, 3756). The present paper shows that BCME can also be extremely dangerous on acute exposure.

A trainee employed in feeding aluminium chloride into a mixing vat containing BCME in methylene chloride added the catalyst too rapidly and the reaction rate increased. Although he was equipped with a breathing mask and rubber protective clothing, the resulting spray of BCME which covered him caused serious harm. Severe irritation of the skin and eyes, with corneal opacity, appeared initially. In the following hours, second- and third-degree burns developed on the affected parts of his body. The optic nerves atrophied and a bilateral lung inflammation, which was resistant to treatment, developed and subsequently led to lung fibrosis and death. Fellow workers who were slightly exposed to the spray also experienced eye irritation.

Following this event, the authors investigated the acute effects of BCME in animals. Citing published data, they point out that the approximate LD_{50}s of BCME are 0·4 ml kg for oral administration to rats and 0·0064 ml/kg for ip dosing in mice. No characteristic signs of poisoning were observed. An atmosphere saturated with BCME vapour at 20 C caused death in all rats exposed for 3 min. Rapid motor activity, laboured respiration, irritation of the skin and mucous membranes, milky corneal opacity and narcosis were observed. Undiluted BCME applied to the shaved dorsal skin of rabbits produced local necrosis within 15 min and a 20-hr application caused a deep tissue necrosis. Instillation of 0·05 ml undiluted BCME into the conjunctival sac of the rabbit caused conjunctival erosion with inflammation and corneal opacity.

A retrospective study of accidents occurring in the factory between 1964 and 1971 revealed 13 incidents in which BCME exposure was involved. Apart from two cases of eye damage involving deep corneal erosion and mild local skin damage, most of the incidents concerned the escape of BCME from leaking conduits or tanks and its subsequent vaporization. Complaints of irritation of the upper respiratory tract and transient headache and nausea were made. No lasting effects, apart from the eye damage, were recorded. Eight cases of lung cancer that may be associated with unknown levels of exposure to BCME are also reported in this paper. Six of these cases came from a group of 18 workers in the experimental technology section, and two of the victims died when comparatively young

(31 and 42 yr). The incidence of lung cancer was considerably higher than the normal, even taking into account the fact that at least six of the eight victims were smokers. The exposure period of 6–9 yr recorded in these eight cases was relatively short, and the latent period before the appearance of the lung disease was about 8–16 yr.

These observations underline the fact that exposure to BCME can represent both a short- and a long-term hazard, and provide further justification for the already effected replacement of industrial processes involving the direct use of BCME (*Cited in F.C.T.* 1974, **12,** 552).

2802. Urinary metabolites as indices of styrene exposure

Ikeda, M., Imamura, T., Hayashi, M., Tabuchi, T. & Hara, I. (1974). Evaluation of hippuric, phenylglyoxylic and mandelic acids in urine as indices of styrene exposure. *Int. Arch. Arbeitsmed.* **32,** 93.

It has been suggested (*Cited in F.C.T.* 1971, **9,** 753) that the estimation of urinary mandelic acid and phenylglyoxylic acid might serve as an index of exposure to atmospheres containing styrene. The other main urinary metabolite, hippuric acid, has been considered to be a relatively insensitive index of styrene exposure in man (*ibid* 1968, **6,** 811), since it is normally present in the urine in relatively high and fluctuating amounts. The authors of the study cited above, however, have found a clear increase in hippuric acid excretion in the urine of workers after intensive exposure to concentrations of styrene vapour.

In six male factory workers exposed to 50–200 ppm styrene vapour for two 80-min periods separated by a 200-min interval, there was a marked increase in the urinary levels of phenylglyoxylic and mandelic acids in the 2–5 hr after exposure ceased. A few hours later, the urinary level of hippuric acid reached a peak, returning to pre-exposure levels (the upper limit of which was taken as 1 mg/ml) within 1–2 days. There was no significant increase in the urinary hippuric acid level of workers exposed for 120 min to 4–60 ppm styrene vapour.

A single dose of up to 910 mg styrene/kg body weight dissolved in corn oil was administered ip to rats to investigate the quantitative relationship between styrene intake and the excretion of the urinary metabolites, phenylglyoxylic, mandelic and hippuric acids. No significant rise in the excretion of hippuric acid was observed with a dose of less than 100 mg/kg. The amounts of mandelic and phenylglyoxylic acids excreted in the urine increased linearly with the amount of styrene administered up to doses of 200–250 mg/kg. At 500 mg/kg, the urinary levels of these acids appeared to reach a plateau, while the hippuric acid excretion continued to rise in proportion to the dose of styrene given. Exposure of rats to concentrations of styrene vapour up to 220 ppm for 8-hr periods produced essentially the same pattern of results.

The biological half-life of styrene was estimated as approximately 8 hr, on the basis of measurements of the decrease in metabolite levels in the urine of seven exposed workers. It was suggested by the authors that one of the reasons for the apparent insensitivity of hippuric acid as an indication of exposure to styrene may be the relatively small increment in hippuric acid levels compared with the high and variable non-exposure levels. The peak concentration of hippuric acid was only about twice the upper limit of variation while with both phenylglyoxylic and mandelic acids about a tenfold increase in the upper physiological limit was attained. Nevertheless, because hippuric acid levels continue to rise in response to styrene dosage after the excretion of the other two acids has levelled off, the former acid may be useful as an indicator of severe exposure.

2803. Toxicity profile of dimethyl terephthalate

Krasavage, W. J., Yanno, F. J. & Terhaar, C. J. (1973). Dimethyl terephthalate (DMT): Acute toxicity, subacute feeding and inhalation studies in male rats. *Am. ind. Hyg. Ass. J.* **34,** 455.

Dimethyl terephthalate (DMT), a crystalline solid of low solubility and high melting point, is used as a starting material in the manufacture of linear polyesters. Its possible hazard, particularly when inhaled as dust, has therefore been assessed.

In the male rats, the acute oral LD_{50} of DMT was not reached with doses of 6·5 g/kg. By ip injection the LD_{50} was 3·9 g/kg and death occurred within 48 hr. When applied to the shaved skin of guinea-pigs, DMT produced neither primary irritation nor sensitization. Rats fed a diet containing 1% DMT for 96 days suffered a reduction in mean body weight and feed utilization, but at lower levels (0·5 and 0·25%) DMT feeding was without evident effect. Exposure of rats for 4 hr/day for 58 days to continuous inhalation of fine DMT dust in concentrations of 16·5 or 86·4 mg/m^3 produced no adverse effects, except that the higher concentration caused nose-rubbing, preening and blinking, which continued intermittently throughout exposure. The respirable fraction of the dust used ($\leqslant 5\ \mu$m) accounted for 36% of the total.

No haematological changes were associated with any treatment, and there were no dose-related effects on blood urea nitrogen, serum glutamic–oxalacetic transaminase, ornithine-carbamoyl transferase, serum alkaline phosphatase, blood glucose or serum proteins. No morphological changes were detected in any organs. Male rats that ingested 50–200 mg DMT daily for 115 days showed no impairment in fertility or reproductive capacity, but there was a dose-dependent reduction of weight gain in pups at weaning as a result of parental consumption of 0·5 or 1% DMT in the diet.

2804. Spray-adhesive hazard unproven

Hook, E. B., Hatcher, Norma H., Brinson, Pauline S., Stanecky, Orysia J., Fisher, L., Feck, G. & Greenwald, P. (1974). Negative outcome of a blind assessment of the association between spray adhesive exposure and human chromosome breakage. *Nature, Lond.* **249,** 165.

The abrupt ban on sales of spray-adhesive products by the US Federal Consumer Product Safety Commission last August (*Federal Register* 1973, **38,** 22569, 23355 & 25216) prompted several studies of the data on which the ban was based.

A blind assessment of chromosome breakage in 11 individuals exposed to the sprays and in matched controls failed to substantiate the claim that exposure to the sprays was associated with a five- or six-fold increase in the rate of chromosome breakage in users, a claim linked with the occurrence of birth defects in two children of exposed parents in Oklahoma City. Hook *et al.* (cited above) made a critical examination of some 36 metaphase plates from the peripheral blood of each exposed subject and unexposed control, but found no difference between the two groups in the incidence of chromosomal breaks. A further examination of 72 metaphases from each of four subjects who were among those with the history of heaviest exposure again revealed no significant difference from the corresponding controls. The authors question what evidence of chromosome breakage ought in future to be accepted as a warning signal of possible genetic hazard, pointing out the widespread public concern this official action provoked before it was withdrawn (*Federal Register* 1974, **39,** 3582) and the reported failure of other studies to confirm the initial allegations.

2805. The hazards of glue-sniffing

Shirabe, T., Tsuda, T., Terao, A. & Araki, S. (1974). Toxic polyneuropathy due to glue-sniffing. Report of two cases with a light and electron-microscopic study of the peripheral nerves and muscles. *J. neurol. Sci.* **21,** 101.

Polyneuropathy has been described in industrial workers continually exposed to high concentrations of *n*-hexane (*Cited in F.C.T.* 1973, **11,** 157), the solvent apparently implicated in two instances of glue-sniffing described recently.

Two young men developed polyneuropathy after habitually sniffing glue over periods of 2·5–3 yr. The first man complained of weakness and numbness in both feet and a progressive disturbance of gait, and was eventually unable to walk. He later developed weakness and paraesthesia affecting both hands. The second man also complained of progressive weakness and numbness of the extremities. In the period immediately before their admission to hospital, both patients had indulged in inhaling the vapour from a plastics-bonding glue in which the solvent was 45% *n*-hexane and 55% toluene, but they had previously abused a formula containing 70–100% toluene and a maximum of 30% *n*-hexane. Both men were painters and could conceivably have come into contact with some harmful paint-thinner, but it was supposed from their history that the glue-sniffing habit was primarily responsible for the polyneuropathy. Sural-nerve biopsy showed extensive axonal degeneration especially in the large fibres, without any signs of regeneration even 3 months after the cessation of exposure. It seems significant that the signs of neuropathy developed to a marked degree only after the change to a solvent of high *n*-hexane content. Although toluene, which was probably primarily responsible for the euphoric effects of the glues, has been relatively rarely associated with peripheral-nerve damage, it may have reinforced the effect of the other solvent in these cases.

NATURAL PRODUCTS

2806. Does malnutrition aggravate cassava toxicity?

Pitchumoni, C. S. & Thomas, E. (1973). Chronic cassava toxicity: Possible relationship to chronic pancreatic disease in malnourished populations. *Lancet* **ii,** 1397.

The clinical syndromes of chronic cassava toxicity include degenerative neurological disease and endemic goitre (*Cited in F.C.T.* 1967, **5,** 125) and appear to be attributable to the cyanogenetic glycosides present in the crop.

In southern India, chronic pancreatic disease, which involves pancreatic calcification and diabetes mellitus, is endemic in a population whose diet consists largely of carbohydrate derived from cassava. In Nigeria, a similar syndrome has been observed in the rural population, which consumes large quantities of cassava. It is argued that the combined effect upon the pancreas of an exogenous toxin, such as is found in cassava, and a chronic nutritional deficiency may be greater than that of either factor alone. The development of the toxic syndromes may be related to a shortage of sulphur-containing amino acids, which normally assist the enzyme rhodanase to detoxicate any hydrocyanic acid derived from the diet.

2807. Unravelling hypoglycin teratogenicity

Persaud, T. V. N. (1973). Prevention by leucine of hypoglycin-B induced teratogenesis in the rat. *Expl Path.* **8,** 283.

Hypoglycins-A and -B, toxic amino acid analogues of leucine, have been extracted from unripe ackee fruit (*Blighia sapida*) and are considered responsible for 'vomiting sickness' in people who eat this unripe fruit (*Cited in F.C.T.* 1972, **10,** 277). Hypoglycin-A is a potent teratogen in the rat, possibly because it inhibits fatty acid oxidation, and the paper cited above looks further into the teratogenic action of hypoglycin-B, an effect which has been tentatively attributed to its biodegradation to hypoglycin-A.

Following the intra-amniotic injection of 100 μg hypoglycin-B, alone or in conjunction with its analogue leucine (50 μg) or with riboflavin phosphate (5 μg), into rats on day 14 of pregnancy, foetuses were examined for abnormalities on day 20. Treatment with hypoglycine-B alone produced a high incidence of developmental defects, 84% compared with 5·3% following a saline control injection. The resorption level in both groups was 14%. With the combined hypoglycin-B and riboflavin treatment the abnormality incidence and resorption rate did not differ from those with hypoglycin-B alone, but with the combined leucine and hypoglycin-B treatment the abnormality rate fell to 6·5% and the resorption rate to 9·4%. If, as appears from these results, the teratogenicity of hypoglycin-B is unrelated to the availability of riboflavin to the foetus, it differs in mechanism from the teratogenicity of hypoglycin-A, which appears to act by inhibiting the acyl dehydrogenase flavin-dependent oxidation reaction (*ibid* 1972, **10,** 277). The protection afforded by leucine supports the hypothesis that hypoglycin-B competes with leucine to produce an imbalance of the foetal amino-acid pool, thus altering the pattern of protein synthesis in the foetus.

2808. The guinea-pig defence against monocrotaline

Chesney, C. F. & Allen, J. R. (1973). Resistance of the guinea pig to pyrrolizidine alkaloid intoxication. *Toxic. appl. Pharmac.* **26,** 385.

In a recent article (*Cited in F.C.T.* 1974, **12,** 559), we continued our series of reviews of studies on the toxicity and metabolism of pyrrolizidine alkaloids in a variety of animal species. While the majority of domestic and laboratory animals seem to be susceptible to the hepatotoxic effects exerted by some of these alkaloids, the guinea-pig appears to be an exception. The work described in the paper now under review was designed to investigate this apparent resistance to intoxication and thus to throw further light on the mechanism of susceptibility in other species.

The alkaloid used was monocrotaline, which in rats has an LD_{50} of about 60 mg/kg, a dose causing weight loss, changes in the pulmonary blood supply, hepatic necrosis and megalocytosis. In guinea-pigs, however, a single sc dose up to 240 mg/kg had no clinically or pathologically detectable effects. Comparison of the *in vitro* conversion of monocrotaline by microsomal preparations from the livers of rats and guinea-pigs showed that conversion to the *N*-oxide derivative was effected to a comparable degree by both preparations, the guinea-pig preparation being slightly more active in this respect than that from rat liver. The capacity for dehydrogenation (pyrrole formation), on the other hand, was about 30 times higher in the rat microsomes. Injection of an iv dose of 60 mg monocrotaline/kg to both species, followed after 2 hr by determination of the pyrrole content of the liver homogenates, showed that while pyrrole formation occurred readily in rats, it was negligible in guinea-pigs. Moreover, when the pyrrole derivative itself, dehydromonocrotaline, was injected directly into the mesenteric blood in single doses up to 20 mg/kg, both species showed marked hepatic necrosis and other adverse effects.

These studies thus indicate that the resistance of the guinea-pig to pyrrolizidine alkaloid intoxication is due to the fact that in this species hepatic microsomal activity results principally in the formation of the *N*-oxide derivatives, which are not themselves hepatotoxic and which cannot be converted to pyrroles by the microsomes. The work also provides further confirmation of the views that the hepatotoxic action of these alkaloids depends on their *in vivo* conversion to active pyrroles (*ibid* 1972, **10,** 873) and that the formation of the pyrrole and *N*-oxide derivatives is effected by separate enzymatic pathways.

2809. Citrinin, ochratoxin and bacon

Wu, M. T., Ayres, J. C. & Koehler, P. E. (1974). Production of citrinin by *Penicillium viridicatum* on country-cured ham. *Appl. Microbiol.* **27,** 427.

Krogh, P., Hald, B. & Pedersen, E. J. (1973). Occurrence of ochratoxin A and citrinin in cereals associated with mycotoxic porcine nephropathy. *Acta path. microbiol. scand.* **81,** 689.

Country-cured hams, of the kind popular in the south-eastern parts of the USA, seem to be a happy hunting-ground for those seeking moulds and their toxic metabolites. Following the isolation of ochratoxin-producing *Aspergillus ochraceus* (*Cited in F.C.T.* 1974, **12,** 287) and sterigmatocystin-producing *A. versicolor* (*ibid* 1974, **12,** 797), for example, comes a report of the production of citrinin by all seven strains of *Penicillium viridicatum* isolated from country-cured ham and subsequently cultured on potato-dextrose broth or on ham slices incubated under controlled conditions.

Growth of the isolated moulds was poor at or below 15°C on both substrates and citrinin was detectable only after an incubation period of 21 days, but at 25–30°C production of the toxin increased sharply after day 14 of incubation. After incubation at 25°C for 21 days, citrinin levels of 184–441 μg/100 ml potato-dextrose broth and 225–658 μg/30-g slice of ham were produced by the seven strains of *P. viridicatum*. The authors point out that since country-cured hams of the kind studied are often aged for 4–8 months at 21–32°C, the production of citrinin during this process must be considered a possibility. The advisability of storing such ham at a low temperature is also indicated by these results.

Citrinin has been shown to be nephrotoxic in some animal species, including dogs (Carlton *et al. Fd Cosmet. Toxicol.* 1974, **12,** 479) and pigs, and has been identified as a probable cause of a porcine nephropathy that is a particular problem in some areas of Denmark (*Cited in F.C.T.* 1972, **10,** 117). However, the strain of *P. viridicatum* Westling isolated from cereals used as feed for bacon pigs and shown to produce citrinin also produces another nephrotoxin, ochratoxin A. The second paper cited above presents evidence to support the contention that ochratoxin A is the main cause of Danish porcine nephropathy, with citrinin playing a minor role.

Thin-layer chromatography studies, backed up by the synthesis of derivatives and nuclear magnetic resonance spectroscopy, were carried out on extracts of samples of cereals used as pig-feed in areas with a high incidence of porcine nephropathy. These revealed the presence of ochratoxin A in 58% of the samples and of citrinin in 9%. All samples containing citrinin also contained ochratoxin. Only 6% of control samples collected from batches of high-quality barley were found to contain ochratoxin, in all cases at levels below 190 ppb ($b = 10^9$). In the contaminated feed samples, the maximum concentration of ochratoxin was 27·5 ppm and about half contained more than 200 ppb, the level considered to be the minimum that will damage the kidney tubules in pigs exposed for 3–4 months. The levels of citrinin found in the few affected samples, however, did not exceed 2 ppm.

while levels of 200–400 ppm in feed have been found to be required to induce kidney damage during an exposure period of 1–2 months. While citrinin may be involved in some cases of nephropathy in Danish pigs, ochratoxin A thus appears to be a more important factor. Apart from its adverse effect on the bacon industry, there is the additional problem that ochratoxin residues have been found in the tissues and organs of pigs fed on the contaminated feeds.

2810. Foetotoxic and teratogenic effects of rubratoxin B

Hood, R. D., Innes, Janice E. & Hayes, A. W. (1973). Effects of rubratoxin B on prenatal development in mice. *Bull. env. contam. & Toxicol. (U.S.)* **10,** 200.

Rubratoxin B, a metabolite of *Penicillium rubrum* and *P. purpurogenum*, has been associated with foetotoxic and teratogenic effects in mice (*Cited in F.C.T.* 1974, **12,** 287). Further details of this aspect of its toxicity are described in the paper cited above.

Albino mice were given a single ip injection of rubratoxin B in a dose of 0·4, 0·6, 0·9 or 1·2 mg/kg on one of days 6–12 of gestation, and foetuses were examined on day 18. Embryonic deaths occurred in all treated animals. The mortality rate correlated with dosage, and was dependent on the day on which the injection was given. Doses exceeding 0·4 mg/kg killed all embryos treated on day 8, but there were some survivors of doses given on other days. Teratogenic effects appeared in foetuses surviving administration of 0·4 mg rubratoxin/kg on day 8, which appeared to be the critical time of gestation. Developmental defects included exencephaly, malformed pinnae or jaws, umbilical hernia and open eye. Most of the foetal abnormalities followed maternal dosage on days 6, 7 or 8. Apart from the 0·6 mg/kg dose given on day 12, and the 0·6 and 0·9 mg/kg doses given on day 9, rubratoxin treatment consistently reduced foetal weight by a significant amount. There was some increase in embryonic mortality in the control groups treated with propylene glycol, the solvent used for administration of the toxin, but the increase was not statistically significant.

2811. Urinary metabolite of sterigmatocystin identified

Thiel, P. G. & Steyn, M. (1973). Urinary excretion of the mycotoxin, sterigmatocystin by vervet monkeys. *Biochem. Pharmac.* **22,** 3267.

Sterigmatocystin, a mycotoxin with a chemical structure similar to that of aflatoxin, has been shown to be hepatocarcinogenic in experimental animals (Purchase & van der Watt, *Fd Cosmet. Toxicol.* 1968, **6,** 555; *idem, ibid* 1970, **8,** 289), and its possible importance in the aetiology of liver cancer has been recognized.

In the present study, the major urinary metabolite of sterigmatocystin was isolated, by adsorption on an Amberlite XAD-2 resin column and subsequent elution with methanol, following oral administration of ^{14}C-labelled sterigmatocystin to the vervet monkey. Treatment of the isolated material with β-glucuronidase and extraction of the product with chloroform resulted in the recovery of over 95% of the urinary radioactivity, indicating that the major metabolite was a glucuronic acid conjugate of either sterigmatocystin or one of its derivatives. Thin-layer chromatography (TLC) of the chloroform-extractable material showed that, while four fluorescent compounds were isolated, 94% of the radioactivity was accounted for by unaltered sterigmatocystin. Parallel separations were carried out on urine from monkeys treated with unlabelled sterigmatocystin.

On the basis of these results, the possibility of using TLC, with or without prior treatment with β-glucuronidase, to separate and estimate the conjugates isolated from human urine is suggested as a direct method of assessing human exposure to sterigmatocystin.

2812. No percutaneous mycotoxin absorption

Purchase, I. F. H. & Steyn, M. (1973). Absence of percutaneous absorption of aflatoxin. *Toxic. appl. Pharmac.* **24,** 162.

Purchase, I. F. H. & Van der Watt, J. J. (1973). Carcinogenicity of sterigmatocystin to rat skin. *Toxic. appl. Pharmac.* **26,** 274.

The first paper cited above describes a study carried out to ascertain whether aflatoxin was absorbed percutaneously after application to rat skin, as had previously been suggested (*Cited in F.C.T.* 1970, **8,** 697; *ibid* 1971, **9,** 579).

When 100 μg aflatoxin B_1, dissolved in either dimethylsulphoxide (DMSO) or acetone, was applied to the shaved skin between the shoulder blades of anaesthetized female rats, no aflatoxin was found in the internal organs of the rats 20 or 130 min after the application, and there was no significant loss of toxin from the skin during this period. However, from 20–60% of the applied aflatoxin disappeared in 130 min from the skin of similarly treated conscious rats, and the toxin was detected on the paws and in the stomach and, to a lesser extent, the liver and kidneys of these animals. It seemed, therefore, that the liver lesions found in rats treated dermally with aflatoxin (*ibid* 1971, **9,** 579) were more likely to have been due to removal of the toxin from the skin during grooming and its subsequent ingestion than to percutaneous absorption, for which there was no evidence in this study. The wearing of cardboard collars in the comparable study in rabbits (*ibid* 1970, **8,** 697) would not necessarily have prevented this ingestion, and variations in grooming behaviour among individual animals may have been the reason for the observed variations in the severity of the hepatic lesions in treated rats.

The hepatocarcinogenicity of another mycotoxin, sterigmatocystin, following oral administration to rats has been mentioned in the previous abstract. In the second study cited above, the shaved skin of rats was treated with 1 mg sterigmatocystin in DMSO or acetone twice weekly for 70 wk. No animals had developed skin lesions by wk 39, but in wk 40 one rat treated with sterigmatocystin in DMSO developed a papilloma-like lesion and by wk 70 all rats treated with the toxin had developed either papillomas or squamous-cell carcinomas. Application of sterigmatocystin to rat skin appeared to have no significant effect on survival time. Histological examination of tissue specimens taken at autopsy revealed that liver lesions, consisting of regenerative changes or hepatocellular carcinomas, were present in 17 of the 20 treated animals. The lesions were similar to those described following oral administration of the toxin (Purchase & van der Watt, *Fd Cosmet. Toxicol.* 1970, **8,** 289) and the authors suggest that, following skin application, the sterigmatocystin, like the aflatoxin in the study described above, was not absorbed percutaneously but was ingested in the course of grooming.

2813. Another factor in myocardial infarction?

Davies, D. F., Johnson, A. P., Rees, B. W. G., Elwood, P. C. & Abernethy, M. (1974). Food antibodies and myocardial infarction. *Lancet* **i,** 1012.

Statistical relationships have been established between age, blood pressure, serum cholesterol, smoking habit, body mass and exercise and the incidence of coronary heart dis-

ease. Because this information is of only limited predictive value, other factors have been sought in an effort to improve our understanding of the aetiological factors involved.

In an investigation conducted on 216 patients who had had a myocardial infarction and on 144 control patients of the same age and sex, it was found that the proportion of patients with antibodies to dried milk was higher in the group with infarction than in the control group. This was possibly true also for antibodies to egg but not for antibodies to gluten. These differences in proportion were most striking in patients who died within 6 months of infarction. The possession of antibodies to cow's milk protein and egg white soon after infarction seems to be highly predictive of death; in fact mortality was increased almost threefold when either antibody was present.

The authors believe that the relationship between food antigenicity and myocardial infarction is one of cause and effect. This relationship provides a new approach to the study of myocardial infarction based on an immunological hypothesis for coronary heart disease and atheroma. Because food— and, since heating of a protein modifies its antigenicity, processed food in particular—may be an almost unlimited source of antigens throughout life, it is considered advisable to spend some effort in exploring this field.

[It is doubtful whether the findings reported here warrant the postulation of food as a major source of antigens in the production of atheroma and myocardial infarction. The incidence of this disease varies enormously from one geographical area to another, and until some epidemiological data are provided, relating antibody titres to a variety of food proteins and to myocardial infarction, it would be difficult to accept the authors' hypothesis. Moreover, it should be remembered that ordinary cooking procedures in the home still account for the greater proportion of the processing that food undergoes before reaching the dining table.]

COSMETICS, TOILETRIES AND HOUSEHOLD PRODUCTS

2814. Teratogenic effects of hexachlorophene

Kimmel, C. A., Moore, W., Jr., Hysell, D. K. & Stara, J. F. (1974). Teratogenicity of hexachlorophene in rats. Comparison of uptake following various routes of administration. *Archs envir. Hlth* **28,** 43.

Earlier this year (*Cited in F.C.T.* 1974, **12,** 567) we referred to a preliminary report of a study on the possible embryotoxicity of hexachlorophene (HCP) in the rat. That report described a significant increase in foetal malformations and in foetal deaths and resorptions after intravaginal administrations of a large dose of HCP on days 7–10 of gestation. A more detailed report of this and related studies has now been published. Aqueous suspensions of HCP in a concentration of 45, 12 or 3% were administered intravaginally in a dose of 0·2 ml (equivalent to approximately 300, 80 and 20 mg/kg body weight, respectively) on each of days 7–10 of gestation, and the animals were killed on day 20. A 3% commercial HCP product was also tested.

Pregnant rats treated with 45% HCP showed signs of severe toxicity, including weakness, weight loss and diarrhoea, during and for several days after treatment and they also developed a vaginal infection. Similar, but less severe, effects were seen in rats given 12% HCP, but neither the 3% suspension nor the commercial product had any such effects. The incidence of foetal deaths and resorptions was increased significantly only in the group given 45% HCP. This group also showed a significant reduction in foetal weights. The inci-

dence of malformations was clearly dose-related, affecting 44% of the survivors in the group given 45% HCP and 19% in that given 12% HCP, while no malformations were found in the groups given 3% HCP or the commercial product and only 2% of the controls were affected. The commonest malformations were hydrocephaly, anophthalmia, microphthalmia, wavy ribs and urogenital defects.

Other pregnant rats were given a single dose of [^{14}C-methylene]HCP in a dose of 45 mg (10 μCi) intravaginally, orally or dermally on day 10 of gestation. Plasma levels of radioactivity were very high 24 hr after oral treatment but were subsequently similar to those in the vaginally treated group, while levels remained low throughout after dermal application. Apart from high levels in the vagina following administration by that route, tissue levels of radioactivity were similar after vaginal and oral administration and were again considerably higher than those in the dermally-treated group. The highest tissue levels were found in the liver in all groups, while levels in the embryo and maternal brain were significantly lower than in other tissues after oral and vaginal treatment, indicating the existence of blood–brain and placental–embryo barriers for HCP. Within 6 days of vaginal administration, some 33% of the dose of radioactivity was excreted in the faeces, while the urine accounted for only 3%. The faeces have previously been identified as the main route of excretion or orally administered HCP.

2815. Seeing the hair dye through

Frenkel, E. P. & Brody, F. (1973). Percutaneous absorption and elimination of an aromatic hair dye. *Archs envir. Hlth* **27,** 401.

The percutaneous absorption of compounds used in hair dyes is usually low (*Cited in F.C.T.* 1972, **10,** 884). The absorption of HC Blue No. 1 (HCB), a slightly (0·38%) water-soluble nonionic dye prepared by the partial catalytic hydrogenation of 2,4-dinitro-*N*-methylaniline and hydroxyethylation of the product, has been studied in rats and rabbits by the authors cited above.

In one of the experiments, measured areas of the shaved skin of rats and rabbits were painted with a saline solution of 250 μg HCB/ml and allowed to dry. Rats were also given ip and sc doses of 2·5–100 mg HCB for metabolic studies. From all the animals, urine, faeces and bile were collected separately and assayed for HCB. After HCB injection, the cerebrospinal fluid and the faeces of animals whose bile duct had been cannulated contained no detectable dye. The mean total recovery of injected HCB in bile and urine was 82% (range 62–93%), with 90% of the recoverable dye appearing within 6 hr. Thin-layer chromatography of the recovered dye showed that no metabolites of HCB were present.

After dermal application of about 1 mg HCB/cm^2, 80–90% was retained in the skin after 4 hr. In rats, about 1% of the applied dose was recovered from the bile and slightly more from the urine, while in rabbits the mean urinary excretion was 4·5%. Urinary excretion of the dye was more than doubled by occlusion of the treated area with aluminium foil for 4 hr. It was possible to recover more than 90% of an intradermally injected dose of HCB by extraction of skin excised from the area 3 min after the injection.

The working concentration at which HCB is used in hair-dyeing is estimated to result in a level of contact with the scalp of about 0·2 mg/cm^2.

[Perhaps the fact that HC Blue No. 1 is an alkylated and nitrated derivative of *p*-phenylenediamine should be borne in mind in connexion with its use in hair-tinting preparations, even though it appears to be stable in the body.]

CANCER RESEARCH

2816. Methylchrysene carcinogenicity

Hoffmann, D., Bondinell, W. E. & Wynder, E. L. (1974). Carcinogenicity of methylchrysenes. *Science, N.Y.* **183,** 215.

Interest in the possible tumour initiating and carcinogenic activity of chrysene and its methyl derivatives stems mainly from the fact that they are present with benzanthracenes and benzopyrenes in neutral tobacco-smoke subfractions that show a high level of tumour-initiating activity. Early studies (Dunlap & Warren, *Cancer Res.* 1943, **3,** 606) suggested that chrysene itself was marginally carcinogenic and 1-, 4- and 6-methylchrysenes were inactive, while 5-methylchrysene produced a high incidence of sarcomas.

In the present study, chrysene and the six methylchrysenes were synthesized individually to a final purity of >99·9%. Each compound was then assayed for tumour-initiating activity in female mice. Ten doses of 0·1 mg in acetone (total dose 1 mg) were applied on alternate days to the shaved back of each mouse and 10 days after the last application the process of promotion was started. This involved application of 2·5 μg tetradecanoyl phorbol acetate in acetone three times weekly for 20 wk (total dose 0·15 mg). At the same time each compound was tested for carcinogenicity by application of 0·1 mg in acetone to the shaved dorsal skin of mice three times weekly throughout the test. In both studies, benzo[a]pyrene in acetone was used as a positive control and acetone as a negative control.

The 3- and 5-methylchrysenes, especially the latter, were found to be strong tumour-initiators under the conditions studied, while the other five test compounds showed only moderate initiating activity. 5-Methylchrysene also demonstrated a high degree of activity in the first 30 wk of the carcinogenicity study, by which time neither the other five methyl derivatives nor chrysene itself had shown any significant carcinogenicity. Later stages of this study are not reported.

These authors estimate that cigarette smoke contains 4–6 times more chrysene and methylchrysenes than benzo[a]pyrene. The findings in their tumour-initiating study therefore support the idea that the tumour-initiating activity of chrysenes contributes significantly to the carcinogenicity of tobacco smoke, at least to mouse skin. The interim result of the actual carcinogenicity study points to the importance of methylation in position 5 of the chrysene ring and studies on this aspect are continuing.

Fd Cosmet. Toxicol. Vol. 13, p. 165. Pergamon Press. 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:*

Long-term toxicity of indigo carmine in mice. By Jean Hooson, I. F. Gaunt, Ida S. Kiss, P. Grasso and K. R. Butterworth.

Saccharin: Lack of chromosome-damaging activity in Chinese hamsters *in vivo*. By G. F. van Went-de Vries and M. C. T. Kragten.

Short-term toxicity of isoamyl salicylate in rats. By J. J.-P. Drake, I. F. Gaunt, K. R. Butterworth, Jean Hooson, Joan Hardy and S. D. Gangolli.

Studies on degraded carrageenan in rats and guinea-pigs. By P. Grasso, S. D. Gangolli, K. R. Butterworth and M. G. Wright.

Studies on the metabolism of dimethylnitrosamine in the rat. I. Effect of dose, route of administration and sex. By J. C. Phillips, B. G. Lake, Christine E. Heading, S. D. Gangolli and A. G. Lloyd.

Aflatoxin B_1 hydroxylation by hepatic microsomal preparations from the rhesus monkey. By R. I. Krieger, A. S. Salhab, J. I. Dalezios and D. P. H. Hsieh.

A procedure for the extraction and estimation of rubratoxin B from corn. By A. W. Hayes and H. W. McCain.

The absorption, distribution and excretion of [^{14}C]CTAB, a quaternary ammonium surfactant, in the rat. By B. Isomaa.

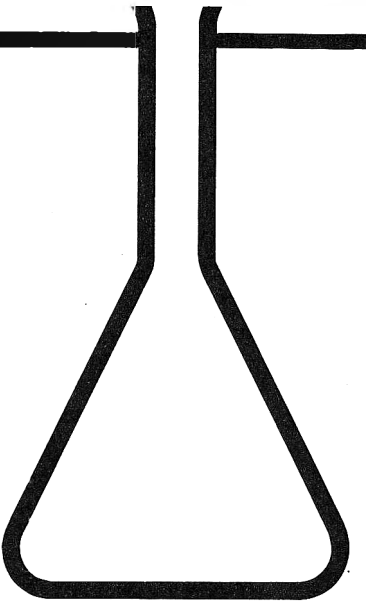
Effect on guinea-pigs of feeding nitrosomorpholine and its precursors in combination with ascorbic acid. By F. J. Akin and A. E. Wasserman. (Short Paper).

Mutagenic evaluation of an alcoholic extract from γ-irradiated potatoes. By H. V. Levinsky and M. A. Wilson. (Short Paper).

Induction of cholangiocarcinoma following treatment of a rhesus monkey with aflatoxin. By T. B. G. Tilak. (Short Paper).

Environmental factors in the origin of cancer and estimation of the possible hazard to man. By H. B. Jones and A. Grendon. (Review Paper).

CURRENT ADVANCES IN ECOLOGICAL SCIENCES



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INFORMATION SECTION

ARTICLES OF GENERAL INTEREST*

TOXICOLOGY: BIBRA ABSTRACTS AND COMMENTS*

*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.

Contents continued]

Aims and Scope

The Journal provides a wide-ranging service of general articles and abstracts on food and cosmetics toxicology. The Journal is primarily intended for the use of food scientists and technologists, manufacturers and administrators who neither read nor have access to the medical, pharmacological or toxicological literature. In addition, the journal will constitute a medium for the publication of reviews and original articles relating to the fields of interest covered by the British Industria Biological Research Association.

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