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Research Section

Short-term Toxicity of Isoamyl Alcohol in Rats

F. M. B. CARPANINI and I. F. GAUNT—*Toxicology*

IDA S. KISS and P. GRASSO—*Pathology*

and

S. D. GANGOLLI—*Biological Chemistry*

British Industrial Biological Research Association, Woodmansterne Road, Carshalton, Surrey, England

(*Received 20 February 1973*)

Abstract—Groups of rats were given daily doses of 0 (control), 150, 500 or 1000 mg isoamyl alcohol/kg body weight for 17 wk. There were no effects associated with treatment in the results of the haematological examinations, serum analyses, urinary cell counts, renal concentration tests or organ weights. A slightly reduced rate of body-weight gain at the highest dose level was shown to be due to a reduced food intake. Two rats given 1000 mg/kg/day died, but histopathological examination showed that these deaths were due to dosing into the lungs and not to any toxic effects of isoamyl alcohol. A female rat given 500 mg/kg/day developed a lipoma, which was not considered to be due to treatment. The other histopathological changes seen were related to mild infections in the animals and not to isoamyl alcohol. The no-untoward-effect level in this study was 1000 mg/kg/day, a level estimated to be 350–400 times the maximum likely intake in man.

INTRODUCTION

Isoamyl alcohol (isopentyl alcohol; 3-methylbutan-1-ol; isobutyl carbinol; $(CH_3)_2 \cdot CH \cdot CH_2 \cdot CH_2OH$) is currently used in the UK as a constituent of flavouring agents.

The Food Standards Committee (1965) in its Report on Flavouring Agents considered isoamyl alcohol but did not include it among the seventeen materials recommended to be prohibited for use in food. It is included (as FEMA no. 2057) in the list of flavourings "generally recognised as safe" by the Expert Panel of the Flavor and Essence Manufacturers' Association, and is permitted by the Food and Drug Administration for use as a synthetic food additive under Sec. 121.1164 of the Code of Federal Regulations. The Council of Europe (1970) suggested an acceptable daily intake for man of 1 mg isoamyl alcohol/kg.

Haggard, Miller & Greenberg (1945) studied the metabolism of primary amyl alcohols in rats and suggested that they were mainly oxidized to the corresponding valeric acids by way of the aldehydes. In fact, in rats given ip doses of 1 g isoamyl alcohol/kg only 0·97 and 0·27% of the dose was excreted unchanged in the expired air and urine respectively. In addition, these authors detected valeraldehyde in the blood of rats after ip doses of *n*-amyl alcohol. The concentration of the alcohol in the blood fell, in 5 hr, from 40 mg/100 ml to a

value reported as undetectable. In rats from which 80% of the liver was removed before dosing, the peak blood levels of the alcohol were higher than in normal rats and the rate of the fall in concentration was slower, suggesting that oxidation occurs in the liver. Haggard *et al.* (1945) also showed that clinical effects of the dose, such as sedation, persisted after both the alcohol and aldehyde were no longer detectable in the blood, and thus appeared to be due to some other metabolite.

In rabbits, a small proportion (9%) of an oral dose of 733 mg isoamyl alcohol/kg was excreted as a glucuronide in the urine within 24 hr (Kamil, Smith & Williams, 1953).

Several workers have studied the acute toxicity of isoamyl alcohol. The oral LD_{50} of mixed isomers of primary amyl alcohol in rats was found to be 5·73 g/kg and the LD_{50} after dermal application in rabbits was 3·22 g/kg (Union Carbide Corporation, personal communication, 1972). Macht (1920) found the lethal dose in cats to be 210 mg/kg when given by iv infusion of a 1% solution in physiological saline at a rate of 2 ml/min. In a similar study in rabbits (2·5% solution infused at 0·5 ml/kg/min), Lehman & Newman (1937) found that the lethal dose was 6·59 g/kg and the minimum dose causing anaesthesia was 850 mg/kg. This species difference may be due to varying rates of metabolism.

There are no published data concerning the prolonged administration of isoamyl alcohol to animals. The present paper describes a short-term toxicity study of the flavouring in rats, carried out as part of the BIBRA safety evaluation programme.

EXPERIMENTAL

Materials. Isoamyl alcohol was supplied by N.V. Chemische Fabriek Naarden, Holland, and complied with the following specification:

> A clear, colourless liquid; purity, 98% (min.); specific gravity (20°/20°C), 0·809–0·815; refractive index (20°C), 1·406–1·412; melting point, −117·2°C; boiling point, 132°C; arsenic, 3 ppm (max); copper and iron, 50 ppm (max); lead, 10 ppm (max); total heavy metals (as lead), 20 ppm (max).

Animals and diet. Rats of the Ash/CSE strain obtained from an SPF breeding colony were fed Spillers' Laboratory Small Animal Diet and given tap-water *ad lib.* The animal rooms were maintained at 20 ± 1°C with a relative humidity of 50–70%.

Experimental design and conduct. Groups of 15 male rats (body weight 80–115 g) and 15 females (body weight 80–105 g) were given isoamyl alcohol by daily oral intubation (7 days/wk) at dose levels of 0 (control), 150, 500 or 1000 mg/kg for 17 wk. In addition, groups of five rats of each sex of similar body weight were given daily doses of 0, 500 or 1000 mg isoamyl alcohol/kg for 3 or 6 wk. The isoamyl alcohol was dissolved in corn oil so that all rats received a dosage volume of 5 ml/kg/day.

The animals were weighed initially, at day 5 and then at weekly intervals up to day 110 of the study, and their consumption of food and water was measured over a 24-hr period preceding the day of weighing.

Urine was collected during wk 3, 6 and 13 of treatment and examined for appearance, microscopic constituents and content of glucose, ketones, bile salts and blood. A concentration test was carried out on the same rats involving the measurement of the specific gravity and volume of urine produced in a 6-hr period of water deprivation. In addition, in the groups examined at 6 and 13 wk the same measurements were made on the urine produced during a 2-hr period following a water load (25 ml/kg) and between 16 and 20 hr after the water load.

The animals were killed by exsanguination from the aorta under barbiturate anaesthesia 24 hr after the final dose of isoamyl alcohol and following an overnight period without food. Portions of the blood obtained were used for haematological examination and serum analyses. The animals were examined for macroscopic abnormalities and the brain, heart, liver, spleen, kidneys, stomach, small intestine, caecum, adrenals, gonads, pituitary and thyroid were weighed. Samples of these organs and of lung, lymph nodes, salivary gland, trachea, oesophagus, aortic arch, thymus, urinary bladder, colon, rectum, pancreas, uterus and skeletal muscle were preserved in 10% buffered formalin. Paraffin-wax sections of these tissues were stained with haematoxylin and eosin for microscopic examination. This examination was confined to half the control rats and those given 1000 mg isoamyl alcohol/kg/day for 17 wk. In addition, the tissues from animals in which abnormalities were seen at autopsy were examined.

Blood was examined for haemoglobin content, packed cell volume and counts of erythrocytes, reticulocytes and total and differential leucocytes. At 17 wk, the counts of reticulocytes were confined to control rats and those given the highest dose level (1000 mg/kg). Serum was analysed for the content of urea, glucose, total protein and albumin and for the activities of glutamic–oxalacetic and glutamic–pyruvic transaminases and lactic dehydrogenase.

Investigation of organ weight changes. Twenty-four weanling male rats (59–70 g body weight) obtained as eight sets of litter-mate trios were used in this study. They were divided into three groups of eight so that one rat from each litter was in each group. One group was given daily doses of 5 ml oil/kg for 3 wk and fed *ad lib.* A second was given 1000 mg isoamyl alcohol/kg/day in 5 ml oil/kg and fed *ad lib.* and the third group was given 5 ml oil/kg but each rat was pair-fed to its isoamyl alcohol-treated litter-mate so that the food consumed by these two groups was equal. The rats were weighed daily and killed after 21 days. An autopsy was conducted during which the brain, heart, liver, spleen, kidneys, stomach, small intestine, caecum and testes were weighed.

RESULTS

One male rat receiving isoamyl alcohol at 1000 mg/kg/day died during wk 8 of the study and one female rat receiving the same level died during wk 10 on test. At autopsy the lung tissue, of both rats, appeared haemorrhagic and there was an excess of fluid in the thoracic cavity. Histopathological examination of the tissues revealed vascular congestion of the lungs, haemorrhage into the alveoli and oil droplets in macrophages. These findings are consistent with accidental dosing by the intratracheal route.

Apart from these two rats, no deaths or abnormalities in behaviour occurred during the study. The rate of body-weight gain was lower than the controls in males given 1000 mg isoamyl alcohol/kg daily, so that at wk 17 the mean body weight of these treated animals was 9% less than that of the controls (Table 1). The differences were statistically significant from wk 9 onwards. There were no statistically significant reductions in body-weight gain at other levels in males or at any of the levels of treatment in females.

There was some reduction in food intake at the highest dietary level of isoamyl alcohol particularly during the early part of the study. However, the differences in mean intakes over the whole period of study were not statistically significant (Table 1). Water consumption was not affected by the treatment.

Table 1. *Mean body weight, food consumption and water consumption of rats dosed with 0–1000 mg isoamyl alcohol/kg/day for 110 days*

Dose level (mg/kg/day)	Body weight (g) at day				Weight gain at day 110 (g)	Food consumption (g/rat/day) at day				Mean consumption (g/rat/day)	Water consumption (ml/rat/day) at day				Mean consumption (ml/rat/day)
	0†	33	68	110		0†	33	68	110		0†	33	68	110	
Males															
0	95	286	420	487	392	14·1	20·7	18·9	19·5	18·5	18·2	27·5	24·9	25·1	24·1
150	95	281	407	469	374	13·9	20·1	17·7	19·8	18·1	18·7	25·7	22·9	23·9	23·7
500	94	288	408	472	378	13·3	22·7	17·5	18·1	18·4	17·9	30·0	27·8	25·8	25·9
1000	93	266	383*	450*	357*	13·0	19·5	18·1	18·7	17·8	19·2	26·2	27·1	26·1	25·1
Females															
0	89	204	258	281	192	12·1	16·9	15·1	16·7	15·4	16·4	21·9	22·0	22·0	21·8
150	90	204	267	293	203	12·5	15·9	15·1	17·2	15·3	18·0	22·5	23·9	21·9	21·5
500	89	202	260	282	193	12·5	15·9	16·5	18·1	15·7	16·7	22·8	24·7	26·5	23·0
1000	88	195	255	284	196	12·8	14·0	14·9	16·6	15·0	16·9	23·2	22·2	23·6	22·7

†Value on the first day of dosing.
Values of body weights are means for 15 animals. Values of food and water consumption are means for three cages of five animals. Although growth and food and water consumption were recorded weekly, only values at monthly intervals are included in the Table.
Figures marked with an asterisk differ significantly (Student's *t* test) from those of controls: $*P < 0.05$.

The only differences from controls seen in the results of the haematological examinations (Table 2) were some isolated increases in packed cell volume, haemoglobin concentration and erythrocyte count at wk 3 and 6. These were not dose-related and were not seen in both sexes at the same examination. There were no significant differences between treated and control animals in the results of serum analyses (Table 3) or urinary cell excretion and renal concentration tests (Table 4). No abnormal constituents were detected in the urine of rats dosed with isoamyl alcohol.

In male rats dosed with 1000 mg isoamyl alcohol/kg/day for 3 wk, the weights of most organs and the terminal body weights were significantly lower than those of controls. The males dosed with 500 mg/kg/day for 3 wk had significantly lower brain, kidney, stomach, small-intestine and testes weights. However, there were no similar differences in the females examined at the same time and when the male organ weights were expressed relative to body weight the only differences seen were in the testes (Table 5). The only difference in the organ weights after 6 wk treatment was a lower pituitary weight in male rats receiving 1000 mg isoamyl alcohol/kg/day, a difference which, again, was not evident when the weights were expressed relative to body weight. The organ weights and relative organ weights were similar in test and control rats after treatment for 17 wk.

At autopsy, there were red patches on the lungs in both sexes at all levels including controls. These were seen mainly at the 3- and 6-wk autopsies. Histopathological examination of the lungs showed only lymphocyte cuffing of the bronchi, the incidence and severity of which were similar in test and control animals. Small cysts in the renal cortex were seen at 17 wk in three control males and one male at the 1000 mg/kg/day treatment level, and microscopically signs of chronic pyelonephiritis were seen. Small testes were seen in three males in each of the treatment levels examined at 3 wk, but the histological appearance of these organs was normal. Small testes were also found in one control male after 17 wk but in this case there was histopathological evidence of testicular atrophy.

One female given 500 mg isoamyl alcohol/kg/day for 17 wk had a mass, 2·5 cm in diameter, close to the right salivary gland, and on histopathological examination, this proved to be a lipoma. No other abnormalities were seen at autopsy or during the histopathological examination.

Investigation of organ weight changes

The rats given daily doses of isoamyl alcohol gained slightly less weight than the controls fed *ad lib.* (Table 6) but the rate of body-weight gain was similarly reduced in the pair-fed control animals. The average food intake of the treated rats was approximately 1 g/day less than that of the *ad lib.* controls. The organ weights of all three groups were closely similar (Table 7).

DISCUSSION

No adverse effects were seen in rats dosed with 150 mg isoamyl alcohol/kg body weight/day for 17 wk.

In view of the histological findings, particularly the presence of oil droplets in the alveolar macrophages, the deaths of the two rats given 1000 mg/kg/day can be attributed not to the toxic action of isoamyl alcohol but to accidental introduction of the oily dose into the lungs.

Table 2. *Haematological values in rats dosed with 0–1000 mg isoamyl alcohol/kg/day for 3, 6 or 17 wk*

Sex and dose level (mg/kg/day)	No. of rats	Hb (g/100 ml)	PCV (%)	RBC (10^6/mm^3)	Retics (% of RBC)	Leucocytes: Total (10^3/mm^3)	Leucocytes: Differential (%) N	E	L	M
Male				**Wk 3**						
0	5	13·4	41	5·68	2·4	5·8	6	0	93	1
500	4	13·9	43	6·09	2·5	5·3	7	1	91	1
1000	4	13·9	43	6·28	2·2	4·9	8	2	88	2
Female										
0	5	13·1	42	5·59	2·9	3·6	8	1	90	1
500	5	13·5	41	6·01	2·3	4·4	8	1	90	1
1000	5	13·5	45*	6·19	1·4	6·0	14	1	83	2
Male				**Wk 6**						
0	5	14·1	43	5·58	1·1	8·7	16	1	82	1
500	3	14·1	42	5·56	1·3	9·3	14	1	83	2
1000	3	14·2	39	5·77	1·6	7·7	23	1	74	2
Female										
0	5	12·1	42	6·67	1·3	8·5	33	2	63	2
500	5	12·9*	44	7·41*	1·2	7·2	36	1	61	2
1000	5	12·0	41	6·68	1·6	5·9	31	1	66	2
Male				**Wk 17**						
0	15	14·3	44	6·84	1·5	5·6	16	1	81	2
150	15	14·0	45	6·56	—	4·8	17	1	81	1
500	15	14·1	44	6·77	—	5·3	15	1	82	2
1000	14	13·9	44	6·78	1·2	5·2	18	1	79	2
Female										
0	15	13·8	42	6·23	1·0	4·2	16	2	81	1
150	15	13·8	43	6·15	—	4·1	14	2	83	1
500	15	13·8	42	6·19	—	2·8	20	2	76	2
1000	14	13·9	43	6·44	0·9	3·9	14	2	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 the numbers of rats shown.
Basophils did not account for more than 0·5% of the leucocytes in any group.
Values marked with an asterisk differ significantly (Student's *t* test) from those of controls: *$P < 0·05$.

Table 3. *Results of serum analyses of rats dosed with 0–1000 mg isoamyl alcohol/kg/day for 3, 6 or 17 wk*

Sex and dose level (mg/kg/day)	No. of rats	GOT (IU)	GPT (IU)	LDH (IU)	Glucose (mg/100 ml)	Urea (mg/100 ml)	Total protein (g/100 ml)	Albumin (g/100 ml)
Male				**Wk 3**				
0	5	50	5·8	945	97	—	6·1	4·2
500	5	47	6·0	875	121	—	6·1	4·1
1000	5	43	6·7	809	103	—	6·0	4·1
Female								
0	5	45	5·8	917	91	14	6·0	4·4
500	5	43	6·2	729	112	18	6·3	4·5
1000	5	48	5·8	948	116	16	6·4	4·4
Male				**Wk 6**				
0	5	44	7·0	790	118	—	6·6	4·4
500	5	38	5·0	849	141	—	6·9	4·4
1000	4	38	9·3	794	119	—	6·4	4·6
Female								
0	5	40	8·2	856	116	19	6·6	4·6
500	5	43	6·8	888	95	20	6·4	4·6
1000	5	38	4·8	807	94	18	6·3	4·6
Male				**Wk 17**				
0	14	39	8·1	863	55	17	7·2	3·8
150	15	39	7·3	823	87	19	7·3	3·8
500	15	39	9·1	827	87	14	7·2	3·7
1000	14	40	6·5	914	55	18	7·0	3·5
Female								
0	15	39	9·7	938	116	15	7·5	4·3
150	14	39	7·9	929	116	19	7·3	4·1
500	15	38	8·5	928	97	15	7·3	3·7
1000	14	40	8·7	928	118	19	7·2	3·9

GOT = Glutamic–oxalacetic transaminase GPT = Glutamic–pyruvic transaminase
LDH = Lactic dehydrogenase

Values are means for the numbers of rats shown.

Table 4. *Mean values of renal concentration/dilution test and urinary cell excretion of rats dosed with 0–1000 mg isoamyl alcohol/kg/day for 3, 6 or 13 wk*

Sex and dose level (mg/kg/day)	No. of rats examined	Cells (10^3/hr)	Concentration test				Dilution test (2 hr)	
			Specific gravity		Volume (ml)			
			0–6 hr	16–20 hr	0–6 hr	16–20 hr	Specific gravity	Volume (ml)
Male				**Wk 3**				
0	5	2·7	1·064	—	0·9	—	—	—
500	5	3·6	1·070	—	1·0	—	—	—
1000	5	3·5	1·068	—	0·8	—	—	—
Female								
0	5	2·2	1·063	—	0·4	—	—	—
500	5	4·8	1·065	—	1·4	—	—	—
1000	5	4·2	1·061	—	1·0	—	—	—
Male				**Wk 6**				
0	5	2·7	1·045	1·062	1·6	0·7	1·011	5·9
500	5	3·0	1·076	1·063	1·4	0·5	1·012	4·8
1000	5	3·0	1·063	1·061	0·8	0·6	1·007	6·0
Female								
0	5	2·0	1·052	1·086	0·6	0·2	1·013	2·6
500	5	2·9	1·066	1·079	0·7	0·4	1·011	2·6
1000	5	3·8	1·070	1·076	0·5	0·6	1·017	2·6
Male				**Wk 13**				
0	12	2·6	1·070	1·071	1·6	1·2	1·008	5·5
150	12	2·5	1·066	1·068	1·7	1·1	1·010	5·5
500	12	3·4	1·061	1·062	1·6	1·5	1·010	5·8
1000	12	2·9	1·061	1·065	1·5	1·2	1·011	5·6
Female								
0	12	1·8	1·063	1·075	0·5	0·5	1·007	5·2
150	12	2·3	1·061	1·071	0·8	0·6	1·008	4·2
500	12	2·3	1·053	1·071	0·4	0·6	1·007	4·6
1000	12	2·2	1·059	1·071	0·5	0·6	1·011	3·7

Results are means for the numbers of rats shown.

Table 5. *Mean relative organ weights of rats dosed with 0–1000 mg isoamyl alcohol/kg/day for 3, 6 or 17 wk*

Sex and dose level (mg/kg/day)	No. of rats examined	Relative organ weight (g/100 g body weight)												Terminal body weight (g)
		Brain	Heart	Liver	Spleen	Kidneys	Stomach	Small intestine	Caecum	Adrenals†	Gonads‡	Pituitary†	Thyroid†	
							Wk 3							
Male														
0	5	0·86	0·41	3·41	0·30	0·85	0·61	3·51	0·33	27	1·26	3·4	7·8	204
500	5	0·94	0·42	3·44	0·34	0·83	0·58	3·45	0·34	29	0·86*	3·4	8·2	180
1000	5	0·98	0·40	3·12	0·29	0·81	0·58	3·13	0·32	24	0·87*	3·4	7·8	165*
Female														
0	5	0·99	0·46	3·39	0·34	0·86	0·59	3·83	0·35	38	62	6·2	9·2	162
500	5	1·07	0·43	3·35	0·33	0·83	0·61	3·86	0·38	40	58	5·5	10·4	153
1000	5	1·08	0·44	3·25	0·32	0·83	0·63	3·94	0·37	38	53	6·6	10·9	148
							Wk 6							
Male														
0	5	0·54	0·33	2·97	0·26	0·66	0·46	2·26	0·31	19	1·00	3·4	5·5	338
500	5	0·57	0·33	3·02	0·29	0·67	0·48	2·28	0·27	19	1·06	3·5	4·9	322
1000	5	0·60	0·33	2·95	0·26	0·67	0·48	2·27	0·29	22	0·94	2·8	5·7	286
Female														
0	5	0·77	0·35	2·97	0·25	0·71	0·58	2·86	0·38	37	59	4·9	8·9	223
500	5	0·77	0·37	2·77	0·32	0·73	0·58	2·67	0·37	37	62	5·4	7·6	221
1000	5	0·76	0·37	2·89	0·34	0·70	0·57	2·77	0·34	35	57	5·4	8·5	223
							Wk 17							
Male														
0	15	0·41	0·28	2·46	0·18	0·56	0·42	1·81	0·22	14	0·77	2·4	4·6	474
150	15	0·42	0·29	2·50	0·18	0·57	0·42	1·88	0·22	14	0·83	2·3	4·5	457
500	15	0·42	0·29	2·48	0·18	0·57	0·44	1·75	0·24	14	0·77	2·4	4·4	464
1000	14	0·44	0·30	2·57	0·21	0·59	0·46	1·76	0·24	15	0·85	2·5	4·4	442
Female														
0	15	0·64	0·33	2·39	0·22	0·59	0·56	2·61	0·31	26	35	4·0	7·1	276
150	15	0·64	0·33	2·40	0·21	0·59	0·55	2·60	0·31	25	37	4·1	6·8	284
500	15	0·67	0·34	2·39	0·22	0·59	0·55	2·62	0·30	28	41	4·2	7·7	274
1000	14	0·65	0·34	2·42	0·21	0·61	0·56	2·56	0·31	28	39	3·7	7·4	273

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

Table 6. *Body weight and mean food intake of male rats given 0 or 1000 mg isoamyl alcohol/kg/day and of pair-fed controls*

Dose level (mg/kg/day)	Diet	Body weight (g) on day								Mean food intake (g/rat/day)
		0	3	6	9	11	14	17	20	
0	*ad lib.*	65	85	106	124	144	157	179	195	15·9
0	Pair-fed	65	83	105	120	130	146	170	184	14·2
1000	*ad lib.*	64	84	103	121	134	152	175	187	14·8

Values are the means for groups of eight rats.

There was a slightly decreased rate of body-weight gain in the male rats at the highest dose level but no similar effect in the females. In the paired-feeding study the body-weight gains of treated and pair-fed controls were similar, both being slightly less than those of control rats given free access to food. This indicates that the failure to gain weight at a normal rate was due to the reduced food intake rather than to a toxic effect of isoamyl alcohol. The mean food intake over the 17-wk experimental period was only marginally reduced, but a consideration of the weekly values showed that this reduction in food intake occurred particularly in the early part of the study. The food intakes of the males given the highest dose level were consistently 5–10% lower than those of controls during the first 6 wk, and the most marked effect occurred after the first dose, when the food intake was reduced by 20%. The reason for such a reduced food intake is uncertain but may be associated with the dosing of relatively high concentrations of the test material (20 g/100 ml at the highest dose level), which could have local irritant effects in the gastro-intestinal tract.

The scattered increases in the haematological indices were not dose-related, were not seen consistently and did not occur after treatment for 17 wk. These differences, which were of marginal statistical significance, were probably random effects due to the small numbers of rats examined rather than an effect of the material administered.

The lower organ weights in the treated male rats killed at wk 3 were accompanied by reduced body weights and, with the exception of the testes, no effects were seen when the organ weights were expressed relative to body weight. In the supplementary study using litter-mate comparisons, these lowered organ weights were not found and the differences in body weight were also less marked. In view of these observations, and as there were no organ-weight changes in females at any time or in males at later examinations, it is likely that the lower organ weights in the males at wk 3 were a reflection of the differences of body weight rather than a direct effect of the isoamyl alcohol.

In the supplementary study, the testes of the treated rats were no smaller than those of the controls. The reduction in testis weight was not seen in rats treated for 6 or 17 wk and there was no histopathological evidence of testicular atrophy. Thus it seems likely that the reduced testis weight seen at wk 3 was also a reflection of the difference in body weight.

Although a single lipoma was found in a female given 500 mg isoamyl alcohol/kg/day, there was no similar finding in other treated animals even at twice this dose level and such lesions have been reported to occur spontaneously with an incidence of 2–3% (Kim, Clifton & Furth, 1960). Thus this isolated finding probably represents the normal incidence and cannot be attributed to treatment.

The no-untoward-effect level for isoamyl alcohol in this study was 1000 mg/kg/day. This is equivalent to an intake of 60 g by a 60-kg adult. From data supplied by seven of the

Table 7. *Organ weights of male rats given 0 or 1000 mg isoamyl alcohol/kg/day and of pair-fed controls*

Dose level (mg/kg/day)	Diet	Organ weights									Body weight (g)
		Brain	Heart	Liver	Spleen	Kidney	Stomach	Small intestine	Caecum	Gonads	
					Absolute weight (g)						
0	*ad lib.*	1·67	0·71	6·29	0·58	1·36	1·07	6·36	0·84	2·03	176
0	Pair-fed	1·67	0·65	5·71	0·57	1·25	1·11	6·27	0·76	2·08	166
1000	*ad lib.*	1·71	0·72	5·86	0·61	1·34	1·13	5·55	0·79	2·04	167
					Relative weight (g/100 g body weight)						
0	*ad lib.*	0·95	0·41	3·57	0·33	0·77	0·61	3·63	0·48	1·15	
0	Pair-fed	1·01	0·39	3·42	0·34	0·75	0·67	3·77	0·46	1·25	
1000	*ad lib.*	1·03	0·44	3·48	0·36	0·81	0·71	3·34	0·48	1·21	

Values are the means for groups of eight rats.

leading flavouring manufacturers it is calculated that the maximum intake of isoamyl alcohol is approximately 170 mg/day. Thus the no-untoward-effect level in the present study is approximately 350–400 times the maximum intake in man.

REFERENCES

Council of Europe (1970). Natural and Artificial Flavouring Substances. Partial Agreement in the Social and Public Health Field. Strasbourg.

Food Standards Committee (1965). Report on Flavouring Agents. HMSO, London.

Haggard, H. W., Miller, D. P. & Greenberg, L. A. (1945). The amyl alcohols and their ketones: Their metabolic fates and comparative toxicities. *J. ind. Hyg. Toxicol.* **27,** 1.

Kamil, I. A., Smith, J. N. & Williams, R. T. (1953). Studies in detoxication. 46. The metabolism of aliphatic alcohols. The glucuronic acid conjugation of acyclic aliphatic alcohols. *Biochem. J.* **53,** 129.

Kim, U., Clifton, K. H. & Furth, J. (1960). A highly inbred line of Wistar rats yielding spontaneous mammosomatotropic pituitary and other tumors. *J. natn. Cancer Inst.* **24,** 1031.

Lehman, A. J. & Newman, H. W. (1937). Comparative intravenous toxicity of some monohydric saturated alcohols. *J. Pharmac. exp. Ther.* **61,** 103.

Macht, D. I. (1920). A toxicological study of some alcohols with especial reference to isomers. *J. Pharmac. exp. Ther.* **16,** 1.

Toxicité à court terme de l'alcool isoamylique chez le rat

Résumé—Pendant 17 semaines on a administré à des groupes de rats des doses quotidiennes de 0 (témoins), 150, 500 ou 1000 mg/kg d'alcool isoamylique. Les résultats des examens hématologiques, des analyses du sérum, des comptages des cellules de l'urine, des tests de concentration rénale et de la pesée des organes n'ont révélé aucun effet imputable au traitement. Une légère diminution du gain de poids chez des animaux qui recevaient la dose la plus élevée a été reconnue imputable à une moindre consommation de nourriture. Deux rats qui avaient reçu 1000 mg/kg/jour sont morts, mais l'examen histopathologique a démontré que la mort était due à l'administration par voie pulmonaire et non à des effets toxiques quelconques de l'alcool isoamylique. Un rat femelle qui recevait 500 mg/kg/jour a présenté un lipome, que l'on n'a pas considéré comme imputable au traitement. Les autres modifications histopathologiques constatées étaient en corrélation avec des infections bénignes contractées par les animaux et non avec l'administration d'alcool isoamylique. Le seuil d'indifférence relevé dans cette étude est de 1000 mg/kg/jour. On estime qu'il équivaut à 350–400 fois la consommation maximale qu'on peut prévoir chez l'homme.

Kurzzeittoxizität von Isoamylalkohol an Ratten

Zusammenfassung—Gruppen von Ratten erhielten 17 Wochen lang tägliche Dosen von 0 (Kontrolle), 150, 500 und 1000 mg Isoamylalkohol/kg Körpergewicht. Es gab keine mit dieser Verabreichung verbundenen Wirkungen in den Ergebnissen der hämatologischen Untersuchungen, Serumanalysen, Zellenzahlen im Urin, Nierenkonzentrationsversuche oder Organgewichte. Eine leicht verminderte Geschwindigkeit der Körpergewichtszunahme bei der höchsten Dosierung wurde als Folge der verminderten Futteraufnahme nachgewiesen. Zwei Ratten, die 1000 mg/kg/Tag erhielten, starben, aber die histopathologische Untersuchung zeigte, dass dies auf die Eingabe in die Lungen und nicht auf toxische Wirkungen des Isoamylalkohols zurückzuführen war. Eine weibliche Ratte, die 500 mg/kg/Tag erhielt, entwickelte ein Lipom, was als nicht auf die Verabreichung zurückzuführen angesehen wurde. Die anderen beobachteten histopathologischen Veränderungen wurden auf milde Infektionen der Tiere und nicht auf Isoamylalkohol zurückgeführt. Die von nachteiligen Wirkungen freie Konzentration bei dieser Untersuchung betrug 1000 mg/kg/Tag, eine Konzentration, die das 350–400fache der maximal wahrscheinlichen Aufnahme durch den Menschen sein dürfte.

Fd Cosmet. Toxicol. Vol. 11, pp. 725–734. Pergamon Press 1973. Printed in Great Britain

Short-term Toxicity of Amyl Cinnamic Aldehyde in Rats

F. M. B. CARPANINI and I. F. GAUNT—*Toxicology*
M. G. WRIGHT and P. GRASSO—*Pathology*
and
S. D. GANGOLLI—*Biological Chemistry*

British Industrial Biological Research Association, Woodmansterne Road, Carshalton, Surrey, England

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Abstract—Amyl cinnamic aldehyde was fed to rats at dietary levels of 0 (control), 80, 400 or 4000 ppm for 14 wk. No differences from controls were seen in the rate of body-weight gain, the consumption of food and water, haematological measurements, serum analyses, urinary cell excretion or renal concentration tests. There were increases in the relative liver and kidney weights of rats fed the highest dietary level, but these were not associated with any histopathological changes. One female rat at each of the two lower dietary levels developed small mammary adenomas, which were thought to be spontaneous. The histopathological changes found were related to the presence of a mild infection rather than to treatment with amyl cinnamic aldehyde. The no-untoward-effect level in this study was 400 ppm (approximately 23 mg/kg/day in males and 36 mg/kg/day in females). This is 500 times the likely maximum intake in man.

INTRODUCTION

Amyl cinnamic aldehyde ($C_6H_5—CH=C(CH_2—(CH_2)_3—CH_3)—CHO$; α-amylcinnamaldehyde; α-pentylcinnamaldehyde; 2-pentyl-3-phenyl prop-2-enal) is a pale-yellow liquid with a floral odour and is used as a flavouring constituent in most categories of food.

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 amyl cinnamic aldehyde was not one of these materials. It is permitted in the USA under Sec. 121.1164 of the Code of Federal Regulations and was classified "generally recognised as safe" (as FEMA no. 2061) by the Expert Panel of the Flavor and Essence Manufacturers' Association. The Council of Europe (1970) suggested a limit of 1 ppm in food.

There are no published data on the metabolism of amyl cinnamic aldehyde. It has been shown that ethyl cinnamic alcohol is oxidized to the corresponding acid but the presence of the substituent on the α carbon prevents β oxidation (Williams, 1959). It seems likely that a similar metabolic route would operate for amyl cinnamic aldehyde.

The oral LD_{50} of amyl cinnamic aldehyde in the rat has been reported by Jenner, Hagan, Taylor, Cook & Fitzhugh (1964) to be 3·73 g/kg. No effect was detected when amyl cinnamic aldehyde was fed to rats for 90 days at 122 ppm in the diet, providing a mean daily intake of 6·5 mg/kg (Oser, Carson & Oser, 1965). The present paper describes a short-term feeding study of the flavouring in rats, carried out as part of the BIBRA safety evaluation programme.

EXPERIMENTAL

Materials. Amyl cinnamic aldehyde was supplied by Bush Boake Allen Ltd. (London), and complied with the following specification: Purity, min. 97%; specific gravity (25/25°C), 0·963–0·968; refractive index (20°C), 1·552–1·559; acid number, max 5·0.

Animals and diet. Rats of the CFE strain, obtained from an SPF breeding colony, were given ground Spillers' Laboratory Small Animal Diet and water *ad lib.* They were housed, five per cage, in an animal room maintained at 21 ± 1°C with a relative humidity of 50–60%.

Loss of amyl cinnamic aldehyde from animal diet. A diet containing 0,000 ppm amyl cinnamic aldehyde was prepared and a sample was placed in a sealed container. The remainder was exposed to the air in an animal room for 48 hr. Methanol extracts of these samples were analysed for amyl cinnamic aldehyde content using a Pye 104 dual-flame ionization gas chromatograph fitted with a 5 ft glass column packed with Celite 545 (100/120 mesh) impregnated with 10% polyethylene glycol adipate. The column temperature was 190°C. Only 1% of the flavouring was lost from the diet exposed to animal-room conditions for 48 hr. As this was not considered to be a significant loss, amyl cinnamic aldehyde was administered in the food, which was prepared every 4–5 days.

Experimental design and conduct

Amyl cinnamic aldehyde was fed to groups of 15 male rats (body weight 100–125 g) and 15 females (body weight 90–120 g) for 14 wk at dietary levels of 0 (control), 80, 400 or 4000 ppm. Additional groups of five rats of each sex were given 0 (control), 400 or 4000 ppm amyl cinnamic aldehyde in the diet for 2 or 6 wk. The animals were weighed initially and then weekly up to wk 14. Food and water consumption were measured over the 24-hr period preceding each weighing.

After the appropriate period of feeding the rats were killed by exsanguination under barbiturate anaesthesia. The blood so obtained was used for haematological studies and serum chemistry. The haematological investigations consisted of measurement of haemoglobin content, packed cell volume, and counts of erythrocytes, total leucocytes and individual types of leucocytes. Reticulocytes were counted in the samples from control rats and those fed the highest level of amyl cinnamic aldehyde (4000 ppm). Serum was analysed for the content of urea, glucose, total protein and albumin and for the activities of glutamic–oxalacetic and glutamic–pyruvic transaminases and of lactic dehydrogenase.

A measurement of urinary concentrating ability was made during the final week of treatment by measuring the specific gravity and volume of urine produced in a 6-hr period of water deprivation. At the same time, samples of the urine were examined for appearance, microscopic constituents and content of cells, glucose, ketones, bile salts and blood. At wk 6 and 13, urine was also collected from the rats over a 2-hr period following a water load of 25 ml/kg and between 16 and 20 hr after the water load.

Each animal was given an autopsy, during which any macroscopic abnormalities were noted and the brain, pituitary, thyroid, heart, liver, spleen, kidneys, adrenals, gonads, stomach, small intestine and caecum were weighed. Samples of these organs and of salivary gland, trachea, aorta, thymus, lymph nodes, urinary bladder, colon, rectum, pancreas, uterus, skeletal muscle and any other tissue that appeared to be abnormal were fixed in 10% buffered formalin. Paraffin-wax sections of these tissues were stained with haematoxylin and eosin for microscopic examination.

RESULTS

One female rat fed diet containing 400 ppm amyl cinnamic aldehyde showed signs of respiratory distress and lost weight rapidly during wk 14. At autopsy consolidation of parts of the lungs was seen and histopathological examination of the tissues revealed pneumonia and an abscess of the renal pelvis.

During the last 3 wk of the study, a subcutaneous mass (0·5–1·0 cm in diameter) was noticed in two female rats (one fed 80 ppm and the other 400 ppm amyl cinnamic aldehyde). At autopsy these masses were seen to be encapsulated and lying free in the subcutaneous space and to consist of firm, white lobular tissue. Histopathological examination showed both to be mammary adenomas.

Apart from these three rats, no abnormalities were seen in the behaviour or appearance of the animals. There were no statistically significant differences between treated and control groups in rate of body-weight gain, food consumption or water consumption (Table 1). The mean intakes of amyl cinnamic aldehyde over the 14-wk period were 6·1, 29·9 and 287·3 mg/kg/day by the males of the groups given 80, 400 and 4000 ppm in the diet and 6·7, 34·9 and 320·3 mg/kg/day by the females. In addition, there were no significant differences between treated and control groups in the results of the haematological examinations (Table 2), serum analyses (Table 3) or urinary cell excretion and renal concentration tests (Table 4).

The only statistically significant differences in the absolute organ weights of treated and control rats were seen after treatment for 6 wk. These consisted of lower stomach weights in the males given 400 ppm amyl cinnamic aldehyde and lower small-intestine weights in the females given 4000 ppm. In the latter case, the difference was not significant when the organ weights were expressed relative to body weight (Table 5). In addition, at the highest dietary level (4000 ppm), there were significant increases in relative liver weights in both sexes after 14 wk treatment and in males after 6 wk. Relative kidney weights were also increased after 14 wk treatment at 4000 ppm.

At autopsy there were red, patchy lungs in three male rats (two at the 80 ppm level and one control) and pale kidneys in some male rats in all groups including controls. Histopathological examination revealed vacuolation of some liver cells, protein casts in the kidney tubules and signs of chronic lung infection. The incidence of these findings was low and was similar in both treated and control rats.

DISCUSSION

There were signs of ill health in a female rat fed amyl cinnamic aldehyde at 400 ppm in the diet for 14 wk but the findings at autopsy and the histopathological examination of the tissues showed this to be due to chronic lung and kidney infection and therefore it could not be related to treatment. Small mammary adenomas were found in two treated female rats. Neither of these rats was from the group fed the highest level of amyl cinnamic aldehyde and tumours of this type are known to occur spontaneously in up to 65% of older female rats (Gaunt, Brantom, Grasso, Creasey & Gangolli, 1972; Gaunt, Carpanini, Grasso, Kiss & Gangolli, 1972; Gaunt, Carpanini, Grasso & Lansdown, 1972). Thus, the presence of these two benign tumours could not be related to treatment with amyl cinnamic aldehyde. Similarly, none of the other histopathological findings in this study could be associated with the intake of diets containing amyl cinnamic aldehyde.

Table 1. *Mean body weights and consumption of food and water for rats fed amyl cinnamic aldehyde at 0–4000 ppm of the diet for 14 wk*

Dietary level (ppm)	Body weight (g) at wk				Weight gain (g) at wk 14	Food consumption (g/rat/day) at wk				Mean food consumption (g/rat/day)	Water consumption (ml/rat/day) at wk				Mean water consumption (ml/rat/day)
	0*	5	9	14		0*	5	9	14		0*	5	9	14	
Males															
0	113	352	435	494	381	17·1	22·1	22·7	19·9	22·1	20·3	28·3	27·6	30·1	26·9
80	118	356	449	509	391	16·9	25·9	23·7	20·5	22·3	20·2	28·7	26·5	29·8	25·8
400	114	353	440	502	388	15·0	23·4	23·5	20·7	21·8	18·3	27·9	28·0	30·5	25·8
4000	116	343	428	490	374	16·6	21·8	22·1	19·7	20·6	20·1	27·7	28·1	29·3	27·5
Females															
0	104	226	267	297	193	15·4	16·5	18·4	14·7	17·8	20·9	23·7	23·3	23·5	23·0
80	106	232	274	305	199	14·9	18·6	20·4	16·1	17·6	22·6	27·0	27·2	27·5	24·9
400	104	238	280	311	207	14·7	19·4	21·6	14·0	18·6	21·6	27·0	27·4	24·5	24·5
4000	104	225	264	292	188	14·3	17·3	19·3	15·1	16·4	20·1	27·2	26·1	25·6	25·0

*First day of feeding.
Body weights are means for 15 animals. Figures for food and water consumption are the means for three cages of five animals.

Table 2. *Haematological values of rats fed 0–4000 ppm amyl cinnamic aldehyde in the diet for 2, 6 or 14 wk*

Sex and dietary level (ppm)	No. of rats	Hb (g/100 ml)	PCV (%)	RBC (10^6/mm^3)	Retics (% of RBC)	Leucocytes: Total (10^3/mm^3)	Leucocytes: Differential (%) N	E	L	M
Male				**Wk 2**						
0	5	13·0	43	6·20	1·80	6·67	9	1	89	1
400	5	13·3	42	6·20	—	5·16	7	0	91	2
4000	5	13·5	43	6·41	1·72	6·09	7	1	90	2
Female										
0	5	12·4	41	5·99	0·90	3·95	10	0	88	1
400	5	12·8	43	6·16	—	3·89	9	1	88	2
4000	5	12·8	43	6·45	1·69	3·47	7	2	89	2
Male				**Wk 6**						
0	5	14·1	44	6·81	0·64	7·52	13	1	84	2
400	5	14·3	45	7·13	—	6·66	14	0	85	1
4000	5	13·9	45	6·94	0·93	6·19	12	1	86	1
Female										
0	5	13·4	43	6·16	1·06	4·33	18	2	79	1
400	5	13·3	42	6·14	—	4·33	16	2	80	2
4000	5	13·6	43	6·18	1·46	2·93	13	2	83	2
Male				**Wk 14**						
0	15	15·3	44	7·15	0·80	6·20	21	2	76	1
80	15	14·9	44	7·10	—	6·46	15	2	82	1
400	15	15·1	44	7·00	—	6·21	17	2	80	1
4000	15	15·1	44	7·32	0·49	6·55	14	1	84	1
Female										
0	15	14·4	43	6·58	0·69	3·55	16	2	81	1
80	15	14·6	44	6·70	—	3·91	18	3	78	1
400	15	14·5	43	6·65	—	3·34	12	2	85	1
4000	15	14·1	43	6·63	0·71	3·21	16	2	81	1

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

Figures are means for the numbers of animals shown.
No inclusions were seen in the erythrocytes. Basophils did not account for more than 0·5% of the leucocytes in any group.

Table 3. *Values of serum analyses for rats treated with 0–4000 ppm amyl cinnamic aldehyde in the diet for 2, 6 or 14 wk*

Sex and dietary level (ppm)	No. of rats	GOT (IU)	GPT (IU)	LDH (IU)	Glucose (mg/100 ml)	Urea (mg/100 ml)	Protein (g/100 ml)	Albumin (g/100 ml)
Wk 2								
Male								
0	5	46	6	897	—	15	5·9	5·5
400	4	43	6	945	—	13	5·8	5·4
4000	5	41	6	875	—	16	5·8	5·5
Female								
0	5	51	7	1169	105	17	—	4·2
400	4	59	6	1280	115	17	—	4·0
4000	5	52	7	971	113	16	—	4·1
Wk 6								
Male								
0	5	56	8	974	81	18	7·1	3·5
400	5	52	7	1071	81	15	7·1	4·0
4000	5	47	12	988	70	18	7·4	3·6
Female								
0	5	46	9	941	75	19	7·3	3·8
400	5	45	6	907	88	19	7·1	4·1
4000	4	46	7	897	50	20	7·3	4·2
Wk 14								
Male								
0	15	46	10	769	141	15	7·4	3·7
80	15	43	9	783	124	16	7·4	4·0
400	15	43	10	794	112	18	7·4	4·0
4000	15	46	9	811	121	19	7·4	4·1
Female								
0	15	45	7	803	115	18	7·5	4·4
80	15	45	8	790	111	19	7·8	4·6
400	15	43	9	845	126	15	7·6	4·2
4000	15	44	7	860	120	15	7·6	4·2

Figures are means for the numbers of rats shown.

Table 4. *Mean values of renal concentration/dilution tests and urinary cell excretion in rats fed amyl cinnamic aldehyde at 0–4000 ppm of the diet for 2, 6 or 14 wk*

Sex and dietary level (ppm)	No. of rats	Cell excretion (10^3/hr)	Concentration test				Dilution test (2 hr)	
			Specific gravity		Volume (ml)		Specific gravity	Volume (ml)
			0–6 hr	16–20 hr	0–6 hr	16–20 hr		
Male			**Wk 2**					
0	5	5·4	1·062	—	1·5	—	—	—
400	5	4·8	1·061	—	2·2	—	—	—
4000	5	3·1	1·066	—	1·2	—	—	—
Female								
0	5	3·7	1·057	—	2·2	—	—	—
400	5	2·5	1·062	—	2·1	—	—	—
4000	5	4·3	1·060	—	1·6	—	—	—
Male			**Wk 6**					
0	5	3·7	1·049	1·068	2·3	0·7	1·010	7·2
400	5	4·0	1·054	1·076	2·9	0·9	1·012	5·5
4000	5	3·9	1·056	1·065	2·5	1·4	1·009	6·4
Female								
0	5	2·6	1·049	1·072	1·4	0·8	1·010	3·2
400	5	2·8	1·037	1·075	1·6	0·6	1·007	3·6
4000	5	3·8	1·045	1·073	1·0	0·7	1·013	2·7
Male			**Wk 14**					
0	12	3·2	1·060	1·072	1·9	1·0	1·011	6·7
80	12	3·6	1·056	1·067	1·7	1·5	1·012	6·0
400	12	3·2	1·062	1·067	1·4	1·4	1·014	5·5
4000	12	3·6	1·060	1·065	1·6	1·4	1·012	6·7
Female								
0	12	2·2	1·062	1·079	0·5	0·3	1·010	5·3
80	12	2·9	1·051	1·068	0·9	0·6	1·011	4·4
400	12	3·8	1·049	1·068	0·7	0·3	1·012	4·9
4000	12	4·1	1·051	1·063	1·2	1·1	1·008	5·3

Results are means for the numbers of rats shown.
Tests for glucose, bile salts, blood and ketones were negative.

Table 5. *Relative organ weights of rats fed amyl cinnamic aldehyde at 0–4000 ppm of the diet for 2, 6 and 14 wk*

Sex and dietary level (ppm)	No. of rats	Relative organ weights (g/100 g body weight)												Terminal body weight (g)
		Brain	Heart	Liver	Spleen	Kidneys	Stomach	Small intestine	Caecum	Adrenals†	Gonads‡	Pituitary†	Thyroid†	
							Wk 2							
Male														
0	5	0·76	0·41	3·33	0·33	0·86	0·60	3·42	0·36	24·2	1·16	3·84	6·99	226
400	5	0·70	0·41	3·32	0·28	0·82	0·52	3·18	0·35	24·5	1·18	3·89	6·97	240
4000	5	0·75	0·40	3·73	0·30	0·87	0·55	3·21	0·34	25·1	1·21	4·02	8·02	229
Female														
0	5	0·94	0·43	3·40	0·34	0·84	0·58	3·45	0·36	38·1	51·9	5·82	9·35	169
400	5	1·00	0·44	3·47	0·34	0·87	0·58	3·37	0·37	37·7	55·1	6·33	8·76	163
4000	5	0·97	0·43	3·51	0·35	0·86	0·57	3·38	0·37	36·6	46·0	5·84	9·52	167
							Wk 6							
Male														
0	5	0·51	0·34	2·77	0·25	0·69	0·44	1·80	0·24	18·6	1·00	3·40	4·68	372
400	5	0·51	0·35	2·80	0·24	0·71	0·39*	1·91	0·23	16·9	0·94	3·56	5·43	369
4000	5	0·54	0·35	3·06*	0·24	0·77	0·46	1·92	0·27	19·4	1·06	3·40	5·65	338
Female														
0	5	0·76	0·35	2·75	0·28	0·67	0·51	2·64	0·38	35·2	59·3	4·51	7·77	223
400	5	0·76	0·33	2·56	0·24	0·65	0·62	2·47	0·32	34·7	41·4	4·41	8·40	213
4000	5	0·76	0·34	2·87	0·28	0·70	0·54	2·31	0·39	36·7	47·7	4·66	8·80	217
							Wk 14							
Male														
0	15	0·40	0·28	2·42	0·17	0·60	0·39	1·62	0·24	13·7	0·77	2·34	4·20	476
80	15	0·38	0·28	2·51	0·18	0·58	0·41	1·62	0·24	12·2	0·72	2·40	4·23	492
400	15	0·39	0·28	2·45	0·18	0·60	0·38	1·63	0·23	13·1	0·79	2·35	4·27	485
4000	15	0·40	0·27	2·66**	0·17	0·64**	0·38	1·67	0·25	13·5	0·79	2·44	4·48	467
Female														
0	15	0·64	0·32	2·20	0·22	0·58	0·49	2·20	0·33	24·7	45·0	4·73	7·07	280
80	15	0·61	0·31	2·20	0·23	0·58	0·49	2·25	0·32	25·3	39·3	4·31	7·26	289
400	15	0·61	0·33	2·25	0·22	0·60	0·48	2·17	0·32	26·0	44·7	4·77	6·60	291
4000	15	0·63	0·32	2·35*	0·24	0·61	0·51	2·15	0·36	25·7	47·4	4·70	6·90	276

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

The lower stomach and small-intestine weights found at wk 6 were probably not due to treatment with amyl cinnamic aldehyde. In both cases they were seen only in one sex and were not evident after longer periods of exposure. In addition, in the case of the reduced stomach weight at 400 ppm no similar effect was found at ten times this dietary level.

The increased relative liver weight seen in rats after exposure for 6 and 14 wk to 4000 ppm amyl cinnamic aldehyde in the diet was not accompanied by any histological abnormality. Such liver enlargement without histological change has been described previously for many compounds, including BHT (Gilbert & Golberg, 1965). In some cases, it has been ascribed to a stimulation of the microsomal processing enzymes (Golberg, 1967). It may be argued that such an enlargement, due to a physiological stimulation, may not be of toxicological significance. However, in the present case there is no evidence to confirm that such a stimulation was responsible for the enlarged liver and so, until more information is available, the liver enlargement must be regarded as an untoward effect.

Similarly the increase in relative kidney weights in male rats fed amyl cinnamic aldehyde at 4000 ppm was not accompanied by any histopathological abnormalities or significant changes in the measurement of renal function but must, at present, be attributed to treatment with the flavouring.

The no-untoward-effect level for amyl cinnamic aldehyde from these studies is 400 ppm in the diet and the toxicological significance of the changes at ten times this level are uncertain. A dietary intake of 400 ppm corresponded to an average daily intake of approximately 30 mg/kg body weight in males and 35 mg/kg in females or to an intake of 1·4–2·2 g/day for a 60-kg human adult and correspondingly less in children. From data supplied by seven of the leading flavouring manufacturers, the calculated daily intake of amyl cinnamic aldehyde by man is between 1·30 and 2·65 mg. The no-effect level found in this study is therefore at least 500 times the estimated intake in man.

REFERENCES

Council of Europe (1970). Natural and Artificial Flavouring Substances. Partial Agreement in the Social and Public Health Field. Strasbourg.

Food Standards Committee (1965). Report on Flavouring Agents. HMSO, London.

Gaunt, I. F., Brantom, P. G., Grasso, P., Creasey, Margaret & Gangolli, S. D. (1972). Long-term feeding study on Chocolate Brown FB in rats. *Fd Cosmet. Toxicol.* **10,** 3.

Gaunt, I. F., Carpanini, F. M. B., Grasso, P., Kiss, Ida S. & Gangolli, S. D. (1972). Long-term feeding study on Black PN in rats. *Fd Cosmet. Toxicol.* **10,** 17.

Gaunt, I. F., Carpanini, F. M. B., Grasso, P. & Lansdown, A. B. G. (1972). Long-term toxicity of propylene glycol in rats. *Fd Cosmet. Toxicol.* **10,** 151.

Gilbert, D. & Golberg, L. (1965). Liver response tests. III. Liver enlargement and stimulation of microsomal processing enzyme activity. *Fd Cosmet. Toxicol.* **3,** 417.

Golberg, L. (1967). The amelioration of food. The Milroy Lectures. *J. R. Coll. Phycns Lond.* **1,** 385.

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.

Oser, B. L., Carson, S. & Oser, Mona (1965). Toxicological tests on flavouring matters. *Fd Cosmet. Toxicol.* **3,** 563.

Williams, R. T. (1959). *Detoxication Mechanisms. The Metabolism and Detoxication of Drugs, Toxic Substances and Other Organic Compounds.* 2nd ed., p. 318. Chapman & Hall Ltd., London.

Toxicité à court terme de l'aldéhyde amyl-cinnamique

Résumé—Des rats ont consommé de l'aldéhyde amyl-cinnamique pendant 14 semaines à raison de 0 (témoins), 80, 400 ou 4000 ppm du régime. Aucune différence par rapport aux animaux témoins n'a été constatée en ce qui concerne le gain de poids, la consommation de nourriture et d'eau, les résultats des analyses du sang et du sérum, l'excrétion urinaire de cellules et les tests de concentration rénale. Les poids relatifs du foie et des reins ont augmenté chez les animaux qui recevaient les plus fortes doses, mais ces augmentations n'allaient pas de pair avec de quelconques modifications histopathologiques. Dans chacun des groupes qui recevaient les deux doses les plus faibles, une femelle a présenté de petits adénomes mammaires, que l'on croit cependant être spontanés. Les modifications histopathologiques constatées étaient en corrélation avec la présence d'une légère infection plutôt qu'avec le traitement à l'aldéhyde amyl-cinnamique.

Le seuil d'indifférence relevé ici est de 400 ppm (environ 23 mg/kg/jour chez les mâles et 36 mg/kg/jour chez les femelles). Il est 500 fois plus élevé que la consommation maximale probable chez l'homme.

Kurzzeittoxizität von Amylcinnamylaldehyd an Ratten

Zusammenfassung—Amylcinnamylaldehyd wurde 14 Wochen lang an Ratten in Konzentrationen von O (Kontrolle), 80, 400 oder 4000 ppm im Futter verfüttert. Es wurden keine Unterschiede zu den Kontrolltieren hinsichtlich der Körpergewichtszunahme, des Futter- und Wasserverbrauchs, der hämatologischen Bestimmungen, der Serumanalysen, der Zellenausscheidung mit dem Urin und der Nierenkonzentrationsversuche beobachtet. Es gab Zunahmen des relativen Leber- und Nierengewichts von Ratten, welche die höchste Konzentration im Futter erhielten, aber diese Zunahmen waren nicht mit histopathologischen Änderungen verbunden. Eine weibliche Ratte bei jeder der zwei niedrigsten Konzentrationen entwickelte kleine mammäre Adenome, aber diese wurden für spontan gehalten. Die festgestellten histopathologischen Veränderungen wurden mit dem Vorhandensein einer milden Infektion und nicht mit der Verabreichung von Amylcinnamylaldehyd in Verbindung gebracht.

Die von schädlichen Wirkungen freie Konzentration bei dieser Untersuchung betrug 400 ppm (annähernd 23 mg/kg/Tag bei männlichen und 36 mg/kg/Tag bei weiblichen Tieren). Das ist das 500fache der maximal zu erwartenden Aufnahme beim Menschen.

Fd Cosmet. Toxicol. Vol. 11, pp. 735–746. Pergamon Press 1973. Printed in Great Britain

Long-term Toxicity of Sodium Cyclamate in Mice

P. G. BRANTOM and I. F. GAUNT—*Toxicology*

and

P. GRASSO—*Pathology*

British Industrial Biological Research Association, Woodmansterne Road, Carshalton, Surrey, England

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Abstract—Sodium cyclamate was fed to groups of 30 male and 30 female mice at dietary levels of 0·7, 1·75, 3·5 or 7·0%, with a group of 60 males and 60 females serving as controls. There were slight reductions in the rate of body-weight gain during the last 6 months of the study in the female mice given diets containing 0·7–3·5% cyclamate but this was not thought to represent a toxic effect. A mild anaemia was found in the mice given 7·0% cyclamate in their food. There were no effects attributable to treatment in respect of mortality, organ weights or the incidence of histological changes, including tumours. Particular attention was paid to the histopathology of the urinary bladder but there were no changes related to treatment. It was concluded that sodium cyclamate does not exert a carcinogenic effect in mice at dietary levels up to 7·0% and that the no-untoward-effect level in this study was 3·5%.

INTRODUCTION

Sodium cyclamate (sodium cyclohexylsulphamate; $C_6H_{11}NHSO_3Na$) was permitted in the UK for use as a sweetening agent in soft drinks from 2 June 1965 (The Soft Drinks Regulations 1964, Statutory Instrument 1964, no. 760) and in all foods up to a maximum daily intake of 50 mg/kg body weight from 1 December 1967 (Artificial Sweeteners in Food Regulations 1967, Statutory Instrument 1967, no. 1119). The latter regulation was based on the recommendations of the 1966 report of the Food Additives and Contaminants Committee (FACC) of the Ministry of Agriculture, Fisheries and Food. However, this report made the provision that long-term feeding studies in a species other than the rat should be made available within 5 yr. The second report on cyclamates published by the FACC (1967) considered further data and reaffirmed the recommendations of the first report. The Eleventh Report of the Joint FAO/WHO Expert Committee on Food Additives (1968) also recommended the temporary acceptance of a maximum daily intake for man of 50 mg/kg body weight and suggested that lifespan studies should be carried out within 3 yr using a second species.

On 18 October 1969, following reports of carcinogenicity, the Food and Drug Administration of the USA banned the use of cyclamates as additives for general-purpose foods and beverages. This was followed by a similar announcement by the Ministry of Agriculture, Fisheries and Food on 23 October 1969. Subsequently, similar bans have been announced by most other national governments.

Sodium cyclamate has been shown to have a low order of acute toxicity. The LD_{50} values in mice were 10–15 g/kg after oral intubation, 7 g/kg after ip injection and 4–5 g/kg after im injection. The corresponding values in rats were 12–17, 6 and 3–4 g/kg (Richards, Taylor, O'Brien & Duescher, 1951; Taylor, Richards, Wiegand & Weinberg, 1968).

Rats fed dietary levels of 5% sodium cyclamate (approximately 2·5 g/kg/day) for 2 yr had diarrhoea throughout the study and, compared with controls, had a reduced rate of body-weight gain (Fitzhugh & Nelson, 1950; Fitzhugh, Nelson & Frawley, 1951). Similar effects were reported in rats fed sodium cyclamate in the diet at levels of 5 or 10% for 2 yr (Nees & Derse, 1967). Softening of the stools has also been reported in human subjects receiving 5 g calcium cyclamate/day for 7·5 months (Schoenberger, Rix, Sakamoto, Taylor & Kark, 1953) and in other human studies when high levels of either sodium or calcium cyclamate have been ingested (Berryman, Hazel, Taylor, Sanders & Weinberg, 1968). In all cases the diarrhoea and softening of the faeces disappeared when cyclamate administration was terminated. Studies in rats have shown this laxative effect to be due to the osmotic pressure of unabsorbed cyclamate in the intestine and identical laxative effects were obtained with sodium sulphate (Hwang, 1966).

Stein, Serrone & Coulston (1967) reported vacuolation of liver and kidney cells in monkeys 1 and 48 hr after a single oral dose of 4 or 8 g sodium cyclamate/kg body weight and in rats given 2, 5 or 8 g/kg for 5 days. Similar histopathological changes, together with increased serum transaminase levels, were found in guinea-pigs drinking a 2% solution of sodium cyclamate for 4 months (Göttinger, Hagmüller, Hellauer & Vinazzer, 1968). Histochemical studies on the livers of rats fed for 4 months on a diet containing 5% sodium cyclamate indicated that the activities of the enzymes of the Krebs cycle were reduced while those involved in the breakdown of pentose sugars were increased (J. J. Bernier, unpublished information 1967).

Price, Biava, Oser, Vogin, Steinfeld & Ley (1970) fed a mixture of 10 parts sodium cyclamate with 1 part sodium saccharin at dietary levels providing intakes of 0, 500, 1120 or 2500 mg of the mixture/kg/day to rats for 2 yr. Special attention was paid to the urinary bladders because of indications of bladder carcinogenicity from previous unpublished studies using the technique of pellet implantation. In a total of eight out of 34 animals given sodium cyclamate at the highest level, papillary tumours of the urinary bladder were found. No tumours were found in any other group. During the last 25 wk of the study, cyclohexylamine was added to the diet of half of the animals, to provide a daily dose equivalent to 5% of the cyclamate intake (25–125 mg/kg/day), but this did not appear to affect the incidence of tumours.

Nees & Derse (1967) carried out a reproduction study, mating rats fed 10% sodium cyclamate in the diet. They reported a reduction in litter size and mean weight of offspring, compared with controls. When doses of 50, 100 or 250 mg sodium cyclamate/kg/day were given by stomach tube to females from day 6 to 15 of pregnancy, no adverse effects were seen in the offspring (Fritz & Hess, 1968).

In rats given oral doses of ^{14}C-labelled cyclamate, 94–98% of the dose was recovered unchanged in the urine and faeces within 72 hr. This was not affected by feeding the rats on diets containing up to 10% cyclamate for 22 wk before the metabolic studies (Miller, Crawford, Sonders & Cardinal, 1966; Sonders, Wiegand & Netwal, 1968). The authors concluded that cyclamate was not metabolized. Similar conclusions were reached using ^{35}S-labelled cyclamate in man (Schoenberger *et al.* 1953).

However, it has been shown in rats (Oser, Carson, Vogin & Sonders, 1968; Renwick & Williams, 1969), dogs (Golberg, Parekh, Patti & Soike, 1969; Kojima & Ichibagase, 1966), rabbits and guinea-pigs (Renwick & Williams, 1970), pigs (A. J. Collings, unpublished data 1971), rhesus monkey (Parekh, Goldberg & Golberg, 1970) and man (Davis, Adler & Opsahl, 1969; Kojima & Ichibagase, 1966; Leahy, Wakefield & Taylor, 1967) that a

proportion of cyclamate is metabolized to cyclohexylamine. There is a considerable individual variation in the ability to convert cyclamate to cyclohexylamine. Oser *et al.* (1968) showed that the number of rats in which this occurred was directly related to the dose of cyclamate. A. J. Collings (unpublished data 1971) found that approximately 25% of the human population produced cyclohexylamine from cyclamate with a maximum conversion of 60%. In addition to cyclohexylamine, Golberg *et al.* (1969) identified cyclohexenone, cyclohexenol and *N*-hydroxycyclohexylamine as minor metabolites in volunteers given cyclamate.

The data presented by Renwick & Williams (1969) showed that the degree of conversion increased with length of treatment in rats, rabbits and guinea-pigs. A similar state of affairs in man was reported by Leahy *et al.* (1967). Studies by Renwick & Williams (1969), by A. J. Collings (unpublished data 1971) and by Golberg *et al.* (1969) indicate that it is the flora of the hind gut that carries out the conversion of cyclamate to cyclohexylamine. This ability can be potentiated by short-term feeding with cyclamate (Renwick & Williams, 1969).

As no information was available on the long-term effects of cyclamate in mice and in view of the requirements of the FACC (1966 & 1967) and the Joint FAO/WHO Expert Committee on Food Additives (1968) for studies in a species other than the rat, it was decided to carry out such a study as part of the BIBRA safety evaluation programme. In view of the findings of Price *et al.* (1970), which were published during the course of this study, particular attention was paid to the urinary bladders.

EXPERIMENTAL

Materials. The sample of sodium cyclamate was supplied through an *ad hoc* Committee consisting of representatives of Abbott Laboratories Ltd., Imperial Chemical Industries Ltd. and Laporte Industries Ltd. It was a white crystalline powder, odourless but tasting intensely sweet, and conformed to the following specification, compiled from data supplied by the companies named above and from the British Pharmacopoeia 1963, addendum 1964:

> Soluble at 20°C in 5 parts water, 250 parts 95% alcohol and 25 parts propylene glycol; almost insoluble in chloroform and solvent ether; pH (10% w/v solution) 5·5–7·5; loss on drying not more than 1%; assay, 98·0–101·0% with reference to substance dried at 105°C; sulphate, max 0·12%; cyclohexylamine, max 100 ppm; chloride, less than 0·05% as NaCl; heavy metals (as lead), less than 20 ppm; arsenic, max 2 ppm; dicyclohexylamine, nil.

Animals and diet. Mice of the ASH-CS1 strain (Scientific Products Farm) from an SPF colony were kept in an animal room maintained at 21 ± 1°C with a relative humidity of 50–60%. They were fed reground Oxoid pasteurized breeding diet for rats and mice and given water *ad lib.*

Experimental design and conduct. Groups of 30 mice of each sex were fed diets containing 0·7, 1·75, 3·5 or 7·0% sodium cyclamate for 80 wk. A group of 60 mice of each sex was fed the control diet over the same period. Males were caged individually, while females were caged in groups of five. Body weights were measured on all surviving animals at intervals throughout the experiment.

During wk 14 and 28, blood for haematological investigations was collected from a tail vein of ten mice of each sex from each dietary level. During wk 80–84, blood was collected from a tail vein of all surviving mice immediately before killing. At all three examinations the haemoglobin concentration was measured and a blood smear was prepared and examined for red-cell morphology and the incidence of the various types of leucocyte.

At 14 and 28 wk, the packed cell volume was measured and counts were made of total erythrocytes, total leucocytes and the incidence of reticulocytes.

During the study, mice found *in extremis* were killed, and an autopsy was carried out on these and any that died, unless there was extensive autolysis or cannibalism. At 80–84 wk, all surviving mice were killed by exsanguination under barbiturate anaesthesia following a 24-hr period without food. An autopsy was conducted during which an examination was made for any macroscopic abnormalities and the brain, heart, liver, spleen, kidneys, stomach and small intestine were weighed. Samples of these organs and of pituitary, salivary gland, thyroid, thymus, adrenals, lymph nodes, pancreas, lung, gonads, colon, caecum, rectum, skeletal muscle and any other tissue that appeared to be abnormal were taken and fixed in 10% buffered formalin. The bladders of all mice killed at 80 wk were inflated with 0·5–1·0 ml Bouin's fixative and immersed for 24 hr in this fixative. After this time the bladders were bisected by a median sagittal incision, examined under a low-power (×10) microscope for any gross abnormalities and transferred to 10% formalin.

All tissues were processed in the usual way for wax embedding and sections were stained with haematoxylin and eosin. The bladders from all mice were examined microscopically, as were all tissues from the control animals and from those fed on the diet containing 7·0% sodium cyclamate. At the intermediate levels the microscopic examination was confined to the heart, liver, kidneys and any tissue that appeared to be abnormal at autopsy.

RESULTS

Some mice from each group died during the study (Table 1) but the number dead at any one time was similar for all groups, and there was no correlation between the number of deaths and the dietary level of cyclamate. The gain in body weight during the study (Table 2) and the body weight at autopsy (Table 3) was similar for all groups of male mice. In the females the body weight gains of the treated mice were similar to those of the controls up to wk 52. However, during the last 6 months of the study there were marked losses of weight

Table 1. *Cumulative mortality data for mice fed diets containing 0–7·0% sodium cyclamate for 80 wk*

	Total no. of deaths									
	Males					Females				
Wk no. / Dietary level (%)...	0	0·7	1·75	3·5	7·0	0	0·7	1·75	3·5	7·0
40	2	2	0	0	2	2	1	3	2	3
44	3	2	0	0	2	3	1	3	3	3
48	4	2	0	0	4	4	3	3	3	3
52	4	2	0	0	4	4	3	3	3	3
56	5	2	0	0	5	5	4	4	3	3
60	5	2	0	0	5	7	5	4	3	4
64	8	2	0	2	5	8	7	4	4	4
68	8	4	0	2	6	9	7	7	6	4
72	10	4	1	2	6	9	10	8	6	7
76	10	6	1	5	7	12	11	9	6	8
80	11	7	1	7	8	15	12	10	8	14

Figures are the numbers of mice dead from groups of 30 in the case of the treated animals and of 60 in the case of controls.

in the female mice fed on diets containing 0·7–3·5% sodium cyclamate although the effect was less marked at the highest dietary level (7·0%). The differences between the groups were less obvious at autopsy (Table 3).

Table 2. *Body weights of mice fed diets containing 0–7·0% sodium cyclamate for 80 wk*

Dietary level (%)	Body weight (g) at wk				Weight gain (g) at wk 80
	1	26	52	80	
			Males		
0	19·5	34·0	38·5	39·3	19·8
0·7	21·4	35·7	38·8	40·4	19·0
1·75	20·8	34·4	38·0	38·7	17·9
3·5	22·7	34·4	36·7	39·7	17·0
7·0	17·8	34·3	34·6	37·2	19·4
			Females		
0	18·6	30·7	35·1	35·1	16·5
0·7	18·2	30·7	34·0	28·3	10·1
1·75	18·6	29·2	31·5	25·1	6·5
3·5	17·6	28·8	30·1	26·8	9·2
7·0	16·6	28·6	31·1	30·8	14·2

Values are means for all surviving mice, males being weighed individually and females in groups of ten.

There were no statistically significant differences between the organ weights of the control mice and those fed on diets containing cyclamate (Table 3). The red blood cell counts of males fed 7·0% cyclamate were lower than those of the controls (Table 4) at both 14 and 28 wk. Additionally, at 28 wk the same animals had lower haemoglobin concentrations than the controls, and at 80 wk the haemoglobin concentrations of both sexes fed 7·0% cyclamate were significantly lower than those of the controls.

On gross examination of the urinary bladders, a few mice from most groups, including the controls, were seen to have plaques on the surface of the mucosa (Table 5). Histological examination of the bladders revealed foci of chronic inflammatory cell infiltration, mainly lymphocytes, beneath the epithelium of the bladders from approximately 18% of the animals. The incidence of these findings was not increased by feeding cyclamate. No lesions affecting the epithelium of the bladders were found. However, lymphomatous deposits were seen beneath the bladder epithelium of three female mice (Table 5). These deposits consisted of sheets of cells of uniform cytological appearance replacing the normal architecture of the bladder wall. In all three animals the spleen, lymph nodes and thymus were grossly enlarged and showed lymphomatous changes. Lymphoma cells were also found in the liver and kidney of these three animals. Two of these mice were from the group fed 3·5% sodium cyclamate in the diet and the third was from the group fed 7·0%.

The incidence of histopathological changes in the control and highest treatment (7·0%) groups is shown in Table 6. There were changes in liver, kidney, lung, lymph nodes, adrenals, pancreas, salivary glands and reproductive systems, but in none of these was the incidence or severity adversely affected by cyclamate administration.

Table 3. *Absolute and relative organ weights of mice fed diets containing 0–7·0% sodium cyclamate for 80 wk*

Sex and dietary level (%)	No. of mice examined	Organ							Terminal body weight (g)
		Brain	Heart	Liver	Spleen	Kidneys	Stomach	Small intestine	
		Absolute organ weight (g)							
Male									
0	45	0·44	0·26	1·87	0·11	0·68	0·47	1·57	34·9
0·7	21	0·44	0·26	1·95	0·13	0·70	0·46	1·52	35·1
1·75	27	0·45	0·23	2·05	0·14	0·69	0·43	1·60	35·3
3·5	23	0·45	0·25	2·00	0·11	0·66	0·47	1·67	35·5
7·0	22	0·44	0·24	1·94	0·12	0·73	0·46	1·58	34·4
Female									
0	44	0·45	0·18	1·69	0·16	0·47	0·50	1·48	31·5
0·7	19	0·44	0·16	1·69	0·16	0·45	0·47	1·46	30·2
1·75	18	0·43	0·17	1·48	0·14	0·46	0·46	1·35	28·0
3·5	21	0·43	0·16	1·68	0·13	0·47	0·48	1·38	27·9
7·0	17	0·43	0·16	1·68	0·14	0·42	0·46	1·42	28·4
		Relative organ weight (g/100 g body weight)							
Male									
0	45	1·26	0·74	5·35	0·31	1·94	1·35	4·50	
0·7	21	1·25	0·74	5·54	0·36	1·99	1·31	4·33	
1·75	27	1·27	0·65	5·79	0·40	1·96	1·22	4·51	
3·5	23	1·27	0·70	5·62	0·31	1·85	1·33	4·71	
7·0	22	1·28	0·70	5·63	0·33	2·12	1·34	4·59	
Female									
0	44	1·42	0·57	5·38	0·51	1·48	1·59	4·70	
0·7	19	1·40	0·55	5·61	0·55	1·49	1·56	4·84	
1·75	18	1·54	0·59	5·27	0·50	1·63	1·64	4·82	
3·5	21	1·54	0·58	6·03	0·46	1·70	1·72	4·95	
7·0	17	1·52	0·56	5·91	0·51	1·47	1·62	4·97	

Figures are means for the numbers of mice shown.

Table 4. *Haematological values in mice fed diets containing 0–7·0% sodium cyclamate for 80 wk*

Sex and dietary level (%)	No. of mice examined	Hb (g/100 ml)	PCV (%)	RBC (10^6/mm^3)	Retics (% of RBC)	Leucocytes: Total (10^3/mm^3)	Leucocytes: Differential (%) N	E	L	M
Male				**Wk 14**						
0	10	16·6	44	8·65	0·8	11·42	25	0	75	0
3·5	10	16·0	46	7·95*	0·8	13·43	17	0	82	0
7·0	10	16·1	44	7·61***	0·9	13·61	21	0	79	0
Female										
0	10	16·6	49	8·51	1·5	10·66	13	0	86	1
3·5	10	17·3	49	8·55	0·9	11·13	18	0	86	1
7·0	10	16·9	47	8·32	1·2	8·80	13	0	85	2
Male				**Wk 28**						
0	10	17·8	53	9·66	1·0	17·10	32	1	67	0
3·5	10	18·2	56	9·58	0·7	18·51	32	0	67	0
7·0	10	12·8***	50	8·22***	0·8	18·10	27	0	73	0
Female										
0	10	16·0	47	8·77	1·1	10·33	21	1	77	1
3·5	10	15·4	46	8·57	1·1	11·01	20	1	78	1
7·0	10	16·8	48	8·95	1·2	10·50	17	1	81	1
Male				**Wk 80**						
0	45	14·5	—	—	—	—	35	1	63	1
0·7	20	14·2	—	—	—	—	29	2	68	1
1·75	27	14·2	—	—	—	—	33	1	64	1
3·5	21	14·3	—	—	—	—	32	1	66	2
7·0	22	13·7*	—	—	—	—	31	2	67	1
Female										
0	43	14·8	—	—	—	—	41	1	55	3
0·7	18	14·3	—	—	—	—	41	1	55	3
1·75	16	14·4	—	—	—	—	44	1	53	2
3·5	19	13·8	—	—	—	—	55	1	42	2
7·0	16	13·5**	—	—	—	—	48	1	50	1

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

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

Basophils accounted for no more than 0·5% of the leucocytes in any group.

Table 5. *Lesions of the urinary bladder seen on gross and microscopic examination of mice fed diets containing 0–7·0% sodium cyclamate for 80 wk*

		No. of mice affected									
		Males					Females				
	Dietary level (%)...	0	0·7	1·75	3·5	7·0	0	0·7	1·75	3·5	7·0
Type of lesion	No. of mice examined...	45	21	27	23	22	44	19	18	21	17
Macroscopic											
Plaques		2	0	2	0	0	1	1	1	1	0
Microscopic											
Tumours (lymphomatous deposits)		0	0	0	0	0	0	0	0	2	1
Chronic inflammatory infiltrate in bladder		12	2	4	3	5	11	4	1	1	1

Table 6. *Incidence of histological changes (excluding tumours) found in mice fed diets containing 0 or 7·0% sodium cyclamate for 80 wk*

		No. of mice affected			
		Male		Female	
	Dietary level (%)...	0	7·0	0	7·0
Organ and histological finding	No. of mice examined...	46	24	45	25
Liver					
Focal inflammatory cell infiltration		8	3	4	4
Kidneys					
Focal inflammatory cell infiltration		22	11	13	9
Chronic nephropathy		0	0	4	4
Lungs					
Lymphocytic infiltration in peribronchial tissues and lung parenchyma		0	0	3	2
Lymph nodes					
Enlarged, reactive		14	1	8	6
Adrenals					
Fibrous thickening of capsule and atrophy of zona glomerulosa		0	0	9	5
Sparse lymphocytic infiltration		1	0	0	1
Pancreas					
Foci of lymphocytic infiltration		2	0	3	0
Salivary glands					
Foci of lymphocytic infiltration		0	1	3	1
Ovaries					
Cystic		—	—	4	2
Uterus					
Cystic endometrium		—	—	4	2

The numbers of tumours found are shown in Table 7. Tumours of the lung, adrenals, testes, reticulo-endothelial system and subcutaneous tissue were found in control mice. Some of these tumours also occurred in the treated mice but in no case was the frequency of their occurrence significantly greater than in the controls. Four adenocarcinomas of the kidney, all in male mice (one fed 0·7%, two fed 3·5% and one fed 7·0% cyclamate) and two adenocarcinomas of the mammary gland (one in a male given 3·5% cyclamate and one in a female given 7·0%) were found without similar findings in the control group.

Table 7. *Incidence of tumours in mice fed diets containing 0–7·0% sodium cyclamate for 80 wk*

		No. of mice affected									
		Male					Female				
	Dietary level (%)...	0	0·7	1·75	3·5	7·0	0	0·7	1·75	3·5	7·0
Organ and type of tumour	No. of mice examined...	46	21	27	23	24	45	19	18	21	25
Lungs											
Adenoma		15	5	6	1	7	6	2	6	6	3
Adrenals											
Adenoma		2	0	0	0	0	0	0	0	0	0
Testes											
Interstitial-cell tumour		1	0	0	0	0	—	—	—	—	—
Kidneys											
Adenocarcinoma		0	1	0	2	1	0	0	0	0	0
Reticulo-endothelial system											
Lymphosarcoma		7	2	2	2	0	3	2	3	4	6
Reticulum-cell sarcoma		1	1	1	0	0	0	2	1	1	0
Mammary tissue											
Adenocarcinoma		0	0	0	1	0	0	1	0	0	0
Subcutaneous tissue											
Sarcoma		0	0	0	0	0	1	0	0	0	0

DISCUSSION

The reduced rate of body-weight gain seen in female mice receiving diets containing 0·7–3·5% sodium cyclamate was not seen in the males on the same levels of treatment, or in females on the highest dosage level (7·0%). In addition, when the mice were weighed at autopsy, up to 4 wk after the 80-wk weighing, the differences between the groups were less marked. Thus, this temporary loss of weight in some of the groups cannot be attributed to the feeding of cyclamate, although its cause is, at present, unknown. On the other hand, the lower red cell counts and haemoglobin concentrations seen in all mice fed 7·0% sodium cyclamate must be considered as an effect of the treatment.

Although particular attention was paid in this study to the fixation and examination of the urinary bladders, the only histopathological finding in this organ was the presence of

lymphomatous deposits under the mucosa in three mice. In addition it was noticed that there were extensive lymphomatous changes in many of the tissues in these three mice and the kidneys showed particularly extensive areas of lymphomatous deposition. This involvement of the kidneys may, to some extent, have influenced the deposition in the bladder wall. Thus the lymphomatous changes seen in the bladder appear to be associated with the presence of widespread lymphoma, which is a common finding in mice (Tucker & Baker, 1967). In this study the incidence of lymphoma was not affected by the feeding of cyclamate.

The finding of four renal adenocarcinomas in treated male animals without any similar lesions in the controls might, at first sight, suggest a carcinogenic effect on the kidneys, particularly as it is generally considered that spontaneous renal tumours are rare in mice (Cloudman, 1956; Dunn, 1967). However, this general statement does not appear to be true for the strain of mice used in this study. Data were provided by Shell Research Ltd. (Dr. E. Thorpe, personal communication 1971) for the Shell/CF1 mouse from which the strain used in this study was derived. These data indicate that renal tumours occur mainly in males, the incidence of carcinoma in the control groups of a number of long-term studies varying between 2 and 6%. In the present study the renal tumours were confined to males and the incidence of carcinoma exceeded 6% only in the 3·5% group. However, the overall incidence of carcinoma in all treated male mice was approximately 5%, a figure within the control range mentioned above. On the basis of this evidence, it is considered that the renal tumours found in this study represent the natural incidence expected in this strain of mice, rather than any evidence of cyclamate carcinogenicity.

Similarly, the two mammary adenocarcinomas in the treated mice were probably fortuitous since this strain of mice is known to have an incidence of mammary adenocarcinoma of about 0·5% (Dr. E. Thorpe, personal communication 1971) and this figure is similar to the overall incidence in the present study. These tumours are known to occur spontaneously in many strains of mice (Cloudman, 1956; Gaunt, Brantom, Grasso & Kiss, 1972; Grasso, Fairweather & Golberg, 1965; Tucker & Baker, 1967).

The only other tumours found in treated mice were lung adenomas, which occurred with a similar frequency in test and control mice. There was no evidence that the feeding of cyclamate at a level of 7·0% caused any other pathological changes in the mice.

From the results of this study there is no evidence that sodium cyclamate has any carcinogenic potential in mice when fed at levels up to 7·0% in the diet. The no-untoward-effect level in the diet of mice over 80 wk was 3·5% (approximately 5 g/kg/day).

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REFERENCES

Berryman, G. H., Hazel, G. R., Taylor, J. D., Sanders, P. G. & Weinberg, M. S. (1963). A case for safety of cyclamate and cyclamate–saccharin combinations. *Am. J. clin. Nutr.* **21**, 673.

Cloudman, A. M. (1956). Spontaneous neoplasms in mice. In *Biology of the Laboratory Mouse*. Edited by G. D. Snell. p. 168. Dover Publications, Inc., New York.

Davis, T. R. A., Adler, N. & Opsahl, Jeanette C. (1969). Excretion of cyclohexylamine in subjects ingesting sodium cyclamate. *Toxic appl. Pharmac.* **15**, 106.

Dunn, Thelma B. (1967). Renal disease of the mouse. In *Pathology of Laboratory Rats and Mice*. Edited by E. Cotchin and F. J. C. Roe. p. 149. Blackwell Scientific Publications, Oxford.

Fitzhugh, O. G. & Nelson, A. A. (1950). Comparison of the chronic toxicities of synthetic sweetening agents. *Fedn Proc. Fedn Am. Socs exp. Biol.* **9**, 272.

Fitzhugh, O. G., Nelson, A. A. & Frawley, J. P. (1951). A comparison of the chronic toxicities of synthetic sweetening agents. *J. A.m. pharm. Ass. Sci. ed.* **40**, 583.

Food Additives and Contaminants Committee (1966). Report on Cyclamates. HMSO, London.
Food Additives and Contaminants Committee (1967). Second Report on Cyclamates. HMSO, London.
Fritz, H. & Hess, R. (1968). Prenatal development in the rat following administration of cyclamate, saccharin and sucrose. *Experientia* **24**, 1140.
Gaunt, I. F., Brantom, P. G., Grasso, P. & Kiss, Ida S. (1973). Long-term toxicity studies of Chocolate Brown FB in mice. *Fd Cosmet. Toxicol.* **11**, 375.
Golberg, L., Parekh, C., Patti, A. & Soike, K. (1969). Cyclamate degradation in mammals and *in vitro*. *Toxic. appl. Pharmac.* **14**, 654.
Göttinger, E., Hagmüller, K., Hellauer, H. u. Vinazzer, H. (1968). Über die Wirksamkeit des Süssstoffes Zyklamat auf Leberparenchym, korrespondierende Fermente und Gerinnungsfaktoren des Blutes. *Wien. klin. Wschr.* **80**, 328.
Grasso, P., Fairweather, F. A. & Golberg, L. (1965). A short-term study of epithelial and connective tissue reactions to subcutaneous injection of silicone fluid. *Fd Cosmet. Toxicol.* **3**, 263.
Hwang, K. (1966). Mechanism of the laxative effect of sodium sulfate, sodium cyclamate and calcium cyclamate. *Archs int. Pharmacodyn. Thér.* **163**, 302.
Joint FAO/WHO Expert Committee on Food Additives—Eleventh Report (1968). Specifications for the Identity and Purity of Food Additives and their Toxicological Evaluation: Some Flavouring Substances and Non-Nutritive Sweetening Agents. *Tech. Rep. Ser. Wld Hlth Org.* **383.**
Kojima, S. & Ichibagase, H. (1966). Studies on synthetic sweetening agents. VIII. Cyclohexylamine, a metabolite of sodium cyclamate. *Chem. pharm. Bull., Tokyo* **14**, 971.
Leahy, J. S., Wakefield, M. & Taylor, T. (1967). Urinary excretion of cyclohexylamine following oral administration of sodium cyclamate to man. *Fd Cosmet. Toxicol.* **5**, 447.
Miller, J. P., Crawford, L. E. M., Sonders, R. C. & Cardinal, E. V. (1966). Distribution and excretion of ^{14}C-cyclamate sodium in animals. *Biochem. biophys. Res. Commun.* **25**, 153.
Nees, P. O. & Derse, P. H. (1967). Effect of feeding calcium cyclamate to rats. *Nature, Lond.* **213**, 1191.
Oser, B. L., Carson, S., Vogin, E. E. & Sonders, R. C. (1968). Conversion of cyclamate to cyclohexylamine in rats. *Nature, Lond.* **220**, 178.
Parekh, C., Goldberg, E. K. & Golberg, L. (1970). Fate of sodium cyclamate-^{14}C in the rhesus monkey (M. mulatta). *Toxic. appl. Pharmac.* **17**, 282.
Price, J. M., Biava, C. G., Oser, B. L., Vogin, E. E., Steinfeld, J. & Ley, H. L. (1970). Bladder tumors in rats fed cyclohexylamine or high doses of a mixture of cyclamate and saccharin. *Science, N.Y.* **167**, 1131.
Renwick, A. G. & Williams, R. T. (1969). Gut bacteria and the metabolism of cyclamate in the rat. *Biochem. J.* **114**, 78P.
Renwick, A. G. & Williams, R. T. (1970). The metabolism of cyclamate. Proceedings of the First International Sugar Research Conference, Brussels, p. 3.
Richards, R. K., Taylor, J. D., O'Brien, J. L. & Duescher, H. O. (1951). Studies on cyclamate sodium (sucaryl sodium), a new noncaloric sweetening agent. *J. Am. pharm. Ass.* **40**, 1.
Schoenberger, J. A., Rix, D. M., Sakamoto, A., Taylor, J. D. & Kark, R. M. (1953). Metabolic effects, toxicity and excretion of calcium N-cyclohexylsulfamate (sucaryl) in man. *Am. J. med. Sci.* **225**, 551.
Sonders, R. C., Wiegand, R. G. & Netwal, J. C. (1968). Excretion of cyclamate in the rat. *J. Ass. off. analyt. Chem.* **51**, 136.
Stein, A. A., Serrone, D. M. & Coulston, F. (1967). Ultrastructural and biochemical studies of sodium cyclamate. *Toxic. appl. Pharmac.* **10**, 381.
Taylor, J. D., Richards, R. K., Wiegand, R. G. & Weinberg, M. S. (1968). Toxicological studies with sodium cyclamate and saccharin. *Fd Cosmet. Toxicol.* **6**, 313.
Tucker, Mary J. & Baker, S. B. de C. (1967). Disease of specific pathogen-free mice. In *Pathology of Laboratory Rats and Mice*. Edited by E. Cotchin and F. J. C. Roe. p. 787. Blackwell Scientific Publications, Oxford.

Toxicité à long terme du cyclamate de sodium chez la souris

Résumé—Des groupes de 30 souris mâles et 30 souris femelles ont consommé du cyclamate de sodium à raison de 0,7, 1,75, 3,5 ou 7,0% du régime. Un groupe de 60 mâles et 60 femelles faisait office de témoin. Le gain de poids s'est légèrement ralenti pendant les 6 derniers mois de l'expérience chez les femelles soumises aux régimes à 7,0 et à 3,5% de cyclamate. On ne pense pas que ce ralentissement ait trahi un effet toxique. Une anémie bénigne a été observée chez les souris qui consommaient 7,0% de cyclamate. On n'a pas constaté d'effets imputables au traitement en ce qui concerne la mortalité, le poids des organes ou la fréquence d'altérations histologiques, tumeurs comprises. On a accordé une attention particulière à l'histopathologie de la vessie, sans déceler de modifications en corrélation avec le traitement. On conclut que le cyclamate de sodium n'a pas d'effets carcinogènes chez la souris à des doses allant jusqu'à 7,0% du régime et que le seuil d'indifférence s'est situé à 3,5% dans cette expérience.

Langzeittoxizität von Natriumcyclamat bei Mäusen

Zusammenfassung—Natriumcyclamat wurde an Gruppen von 30 männlichen und 30 weiblichen Mäusen in Konzentrationen von 0,7, 1,75, 3,5 und 7,0% im Futter verfütter , wobei eine Gruppe von 60 männlichen und 60 weiblichen Tieren als Kontrolle diente. Während der letzten 6 Monate der Untersuchung wurden leichte Verminderungen der Körpergewichtszunahme bei den weiblichen Mäusen beobachtet, die Futter mit 0,7–3,5% Cyclamat erhielten, aber dies wurde nicht als ein toxischer Effekt angesehen. Eine geringe Anämie wurde bei den Mäusen mit 7,0% Cyclamat im Futter gefunden. Es gab keine der Cyclamatverabreichung zuschreibbaren Wirkungen hinsichtlich der Mortalität, der Organgewichte und der Häufigkeit von histologischen Veränderungen einschliesslich Tumoren. Besondere Aufmerksamkeit wurde der Histopathologie der Harnblase gewidmet, aber es gab keine Veränderungen, die mit der Cyclamatverabreichung in Beziehung standen. Es wurde daraus der Schluss gezogen, dass Natriumcyclamat keine carcinogene Wirkung auf Mäuse bei Konzentrationen bis 7,0% im Futter hat und dass die von nachteiligen Wirkungen freie Konzentration bei dieser Untersuchung 3,5% betrug.

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Effect of Waxy Corn Starch Modification on Growth, Serum Biochemical Values and Body Composition of Pitman–Moore Miniature Pigs*

T. A. ANDERSON, L. J. FILER, JR., S. J. FOMON, D. W. ANDERSEN, R. L. JENSEN and R. R. ROGERS

Department of Pediatrics, University of Iowa, Iowa City, Iowa 52242, USA

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Abstract—Four groups each of eight Pitman–Moore miniature pigs were weaned at 3 days of age and then fed for 25 days on formula diets identical except for the type of carbohydrate. The diets contained thin-boiling waxy corn starch or one of three chemical modifications of this starch (phosphated distarch phosphate, distarch phosphate and hydroxypropylated distarch glycerol). No statistically significant treatment-related effects were observed on growth, biochemical values of blood or serum, or carcass or liver composition.

INTRODUCTION

Starches prepared from a number of sources (e.g. corn, waxy maize, wheat and tapioca) are spoken of as "modified" when they have been chemically treated to introduce substituent groups on to the glucose units and/or cross-links between neighbouring starch molecules. Such starches are commonly used in the manufacture of food products because they provide one or more desirable characteristics such as appropriate viscosity or texture, stability during thermal processing, cold stability and freedom from syneresis (Wurzburg & Szymanski, 1970). In the United States, several commercially prepared infant formulas and approximately 65% of commercially prepared strained and junior foods (Anderson & Fomon, 1971) contain modified food starches.

A recent review by Filer (1971) has cited results of a number of previously unpublished *in vivo* and *in vitro* studies of the metabolism of modified food starches. In addition, a summary of many previously unpublished toxicological reports regarding the feeding of various modified food starches has been prepared by the Joint FAO/WHO Expert Committee on Food Additives (1972).

Because modified food starches are commonly used in infant foods, because little is known of the digestibility of these starches by the neonate and because of the physiological similarities between the human infant and the young pig (Bustad & McClellan, 1966), it seemed of interest to determine whether several different chemical modifications of a waxy corn starch would affect performance, serum biochemical values or body composition of the miniature pig.

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Waxy corn starch (100% amylopectin, the branched-chain starch polymer) is used in the production of a number of modified food starches. Treatment of starch with dilute acid results in a starch of relatively low viscosity, referred to as "thin-boiling' starch. A liquid formula diet can be prepared with such a starch as the sole carbohydrate

Modified food starch contributes as much as 40% of the calories provided by certain infant foods. The contribution of these foods to the diet is generally limited, however, and market research data (Filer, 1971) suggest that modified food starches rarely account for more than 10% of the calorie intake of infants. Diets employed in the present study were formulated to provide 24% of calories from modified food starch, an amount considerably larger than that likely to be consumed by a human infant.

EXPERIMENTAL

Starches and diets

Thirty-two 3-day-old Pitman–Moore miniature pigs were randomly assigned one of four diets (Table 1), provided through the co-operation of Dr. G. A. Purvis, Gerber Products Company, Fremont, Mich. These diets consisted of water, co-precipitated milk protein (Crest-O-Lac, Type CaS8K, Crest Foods Company, Inc., Ashton, Ill.), corn oil, starch, vitamins and minerals. Proximate analyses confirmed that the diets were nearly identical to one another in the distribution of calories from protein, fat and carbohydrate.

Table 1. *Proximate analysis of diets containing unmodified or chemically modified thin-boiling waxy corn starch*

Dietary component	Content (% w/w)* in diet no.			
	1	2	3	4
Water	82·6	82·4	82·4	83·0
Protein	6·2 (27)	6·4 (28)	6·5 (28)	6·2 (27)
Fat	5·1 (49)	4·9 (48)	4·9 (48)	5·3 (51)
Carbohydrate†	5·4 (24)	5·6 (24)	5·6 (24)	4·9 (22)
Ash	0·7	0·7	0·6	0·6
Calcium	0·104	0·106	0·099	0·104
Phosphorus	0·117	0·141	0·114	0·116
Caloric value (kcal/100 g)	93	92	91	93

*Figures in parentheses denote the percentage of total kcal.

†Source of dietary carbohydrate was acid thin-boiling waxy corn starch: unmodified (diet 1); modified with sodium trimetaphosphate cross-links and stabilized with sodium tripolyphosphate (diet 2); modified with phosphorus oxychloride cross-links (diet 3); or modified with epichlorohydrin cross-links and stabilized by hydroxypropylation with propylene oxide (diet 4).

The thin-boiling waxy corn starch was supplied by Mr. O. Wurzburg, National Starch and Chemical Corp., Plainfield, N.J., and the batch used in the control diet (diet 1) also served as the base starch for each of the various chemical modifications used in the other diets (diets 2–4) and prepared through the co-operation of the American Maize Products Co., Roby, Ind., CPC International, Argo, Ill., and A. E. Staley Manufacturing Co., Decatur, Ill. The thin-boiling waxy corn starch was made by treating waxy corn starch with hydrochloric acid in accordance with the Code of Federal Regulations (21 CFR 121.1031(a),

revised 1 January 1972). The resulting starch had a water fluidity of 80. The modified starches were prepared using the thin-boiling starch and the modifying chemicals at levels typical of those used in making the modified food starches commonly used in the baby-food industry. The phosphated distarch phosphate (diet 2) was made in compliance with 21 CFR 121.1031(d) by treatment with 4·8% sodium tripolyphosphate and 0·59% sodium trimetaphosphate (both on a dry weight basis). The residual phosphorus, 0·4%, corresponded to a degree of substitution (DS) of about 0·02. The distarch phosphate (diet 3) was made in compliance with 21 CFR 121.1031(d) by treatment with 0·08% phosphorus oxychloride. The hydroxypropylated distarch glycerol (diet 4) was made in compliance with 21 CFR 121.1031(e) by treatment with 0·009% epichlorohydrin and 4·2% (dry-weight basis) propylene oxide. The resulting cross-linked hydroxypropyl starch had a DS of approximately 0·06.

The content of vitamins and minerals was intended to be that recommended by Schneider & Sarett (1966), but chemical analysis of the diets revealed less calcium and more phosphorus than intended. The calcium content averaged 0·104% (Table 1), approximately 60% of the 0·166% (at 17% total solids) recommended by Schneider & Sarett (1966) and approximately 75% of the estimated requirement published by the National Research Council (Subcommittee on Swine Nutrition, Committee on Animal Nutrition, Agricultural Board and National Research Council, 1968). Phosphorus content was slightly more than 100% of the National Research Council requirement.

Experimental procedure

Each diet was fed *ad lib.* for 25 days to a group of eight pigs. No additional water was provided and records were kept of food intake and spillage. When 28 days old, each pig was weighed and bled from the anterior vena cava. All animals were killed by intracardiac injection of sodium pentobarbitone and immediately eviscerated. Methods used for the biochemical analyses of blood or serum and the procedures for preparing the carcass for chemical analyses have been described in detail elsewhere (Filer, Fomon, Anderson, Andersen, Rogers & Jensen, 1973).

Data were analysed by one-way analysis of variance as described by Snedecor (1956) with tests of multiple comparison according to the method of Duncan (1955).

RESULTS AND DISCUSSION

Performance

As may be seen from Table 2, gains in body weight were similar whether the miniature pigs were fed diets containing the thin-boiling control starch or one of three modified thin-boiling starches as the sole carbohydrate. Because of the viscous nature of the starch-formula diets, considerable feed waste was encountered and it soon became obvious that accurate separation of the spilled feed from the urine and faeces would not be possible. Because of the known high correlation between rate of gain and feed efficiency (Sutherland, 1965), it was decided to limit evaluation of performance to gain in body weight rather than to risk the introduction of erroneous conclusions regarding feed efficiency.

During the course of the experiment, 15 pigs were treated for diarrhoea with erythromycin succinate (Erythrocin® 50 mg/ml; Abbott Laboratories, Inc., North Chicago, Ill. 60664). An intramuscular injection of the medication (0·5 ml/kg) was administered whenever a

Table 2. *Effect of carbohydrate source on body weights of miniature pigs*

Age of pigs (days)	Mean body weights (g) of pigs fed diet no. 1	2	3	4	Standard error of the mean
3	1233	1129	1146	1225	85·2
7	1456	1217	1240	1326	85·7
14	1921	1697	1727	1838	143·3
21	2723	2496	2550	2636	192·1
28	3411	3129	3290	3249	223·8
Gain (days 7–28)...	1955	1912	2053	1920	184·0

Values are means for groups of eight animals.

liquid stool was observed and a repeat injection was given daily until the diarrhoea ceased (usually 2 or 3 days). The diarrhoea was not thought to be related to starch modification since it was spread through all the groups (occurring in three, four, three and five pigs fed diets 1, 2, 3 and 4, respectively) and subsided rapidly after medication. Pigs treated for diarrhoea gained less body weight (85 g/day) between 7 and 28 days of age than did pigs not treated for diarrhoea (101 g/day), but this difference was not statistically significant.

Blood or serum chemical values

No differences due to treatment were observed in any of the chemical values for blood and serum (Table 3). Serum concentrations of cholesterol and triglyceride were markedly lower in the starch-fed pigs than in our previously studied sow-reared pigs (Filer *et al.* 1973). The starch-formula diets were cholesterol-free and contained highly unsaturated fat. By contrast, the cholesterol content of sows' milk has been reported by Elliott, Vander Noot,

Table 3. *Effect of carbohydrate source on biochemical values in the blood or serum of miniature pigs*

Parameter	Mean values for pigs on diet no. 1	2	3	4	Error mean square*
Blood (100 ml)					
Haemoglobin (g)	12·2	12·5	12·7	12·7	3·03
Serum (100 ml)					
Cholesterol (mg)	86	78	76	72	248
Triglyceride (mg)	66	61	56	64†	286
Calcium (mg)	10·1	10·1	9·9†	10·0†	0·35
Phosphorus (mg)	8·3	8·2	7·7	8·3	0·75
Alkaline phosphatase (auto-analyser units)	65·1	63·6	57·4†	55·7	363·52
Urea nitrogen (mg)	27·6	27·8	25·5	24·9	44·20
Total protein (g)	5·1	4·9	5·0†	5·1	0·12
Albumin (g)	3·0	2·9	3·0†	3·2	0·04
Globulin (g)	2·1	2·0	2·0†	1·9	0·06

*The standard error of the mean may be calculated according to the formula $\sqrt{(EMS/N)}$, where EMS is the error mean square and N is the number of observations used in computing the mean.
†Values are means for groups of seven pigs; all others are means for groups of eight.

Gilbreath & Fisher (1971) to be approximately 250 mg/100 g on a dry-matter basis. The fat of sows' milk consists mainly of saturated and mono-unsaturated fatty acids (Bowland, 1966). Data from several investigators have demonstrated that the concentration of cholesterol in the serum of human infants is related to the lipid composition of the infant's diet (Fomon, 1967).

The concentration of calcium in the serum of pigs fed the starch-containing diets was somewhat less than that observed in our previous studies (Filer *et al.* 1973), possibly reflecting the relatively low calcium concentration of the diet. Concentrations of urea nitrogen in the serum of pigs fed the starch-containing diets were greater than anticipated on the basis of our previous study of sow-reared pigs (Filer *et al.* 1973). Although the percentage of calories (27–28%) supplied from protein in the starch-containing diets (Table 1) was slightly greater than that in sows' milk (approximately 26%), concentrations of urea nitrogen in serum averaged approximately 26 and 18 mg/100 ml, respectively. A possible explanation may be offered. In normal animals such as these, urea clearance is likely to vary within rather narrow limits. Thus, plasma (or serum) concentration of urea will remain proportional to the urinary excretion of urea. Urinary excretion of urea, in turn, is proportional to the total excretion of nitrogen, which reflects primarily the quantity of nitrogen absorbed minus the quantity utilized for tissue synthesis. In this context, it is reasonable to assume that the less rapidly growing pigs fed the starch-containing diets would demonstrate greater urinary excretion and greater plasma concentration of nitrogen (and, consequently, of urea).

Organ weights

The treatment had no statistically significant effects on organ weights, expressed as a percentage of body weight (Table 4). All organs appeared to be grossly normal when inspected at autopsy. The liver weight expressed as a percentage of body weight was slightly less than that found at 28 days of age in our previous studies but the weights of other organs expressed as a percentage of body weight were comparable in this and earlier studies.

Table 4. *Effect of carbohydrate source on organ weights of miniature pigs*

Organ	Mean organ weights (% of body weight) of pigs fed diet no.				Standard error of the mean
	1	2	3	4	
Thyroid	0·014	0·015	0·015	0·016	0·0014
Adrenals	0·015	0·013	0·015	0·015	0·0010
Spleen	0·19	0·19	0·17	0·18	0·016
Kidneys	0·78	0·80	0·77	0·77	0·027
Heart	0·51	0·51	0·48	0·49	0·017
Liver	4·04	4·02	4·00	3·85	0·186
Caecum:					
With contents	0·74	0·73	0·75	0·73	0·084
Without contents	0·23	0·24	0·22	0·23	0·016

Values are means for groups of eight pigs.

Carcass composition

The percentage of water was significantly greater in the carcasses of pigs fed diet 2 than in those of pigs fed diets 1 or 3 (Table 5). No other statistically significant effects of treatment on carcass composition were noted. Carcasses of sow-reared Pitman–Moore miniature pigs

(Filer *et al.* 1973) contained at 28 days of age a lower percentage of water and a greater percentage of fat and ash than did those of pigs fed the starch-containing diets. When the percentage composition of the carcasses of sow-reared miniature pigs is compared with that of pigs fed the starch-formula diets on the basis of comparable weight rather than of comparable age, data for the two groups were similar except for the low carcass-ash concentration in pigs fed the starch-formula diets. Thus, under these conditions of study, the body composition of miniature pigs was more closely related to body weight than to age. It would appear that the low calcium content of the starch-containing diets or, possibly the lack of exercise of the caged pigs, was reflected in the low carcass content of ash, particularly of calcium and phosphorus.

Table 5. *Effect of carbohydrate source on carcass composition of miniature pigs*

		Carcass concentration in pigs fed diet no.				Standard error of the mean
		1	2	3	4	
	Carcass weight					
	Wet (g)...	2584	2275	2425	2296	172·9
Component	Fat-free wet (g)...	2132	1924	2006	1949	137·3
		Wet carcass				
Water (%)		66·3	69·3*	66·4	68·4	0·77
Fat (%)		17·5	13·4	16·9	14·6	0·85
Protein (%)		13·6	14·3	13·7	14·1	0·50
Ash (%)		2·6	3·0	2·9	2·9	0·14
Minerals (mg/100 g):						
Calcium		555	722	710	662	60·4
Phosphorus		464	536	506	521	25·1
Potassium		244	244	237	239	2·8
Sodium		131	141	136	148	3·1
Magnesium		33	29	28	29	1·4
		Fat-free wet carcass				
Water (%)		80·3	80·0	80·0	80·1	0·54
Protein (%)		16·4	16·5	16·5	16·5	0·56
Ash (%)		3·2	3·5	3·5	3·4	0·16
Minerals (mg/100 g):						
Calcium		672	835	853	775	70·3
Phosphorus		562	618	609	610	29·0
Potassium		295	282	286	280	4·0
Sodium		159	162	164	173	2·7
Magnesium		40	33	33	34	1·8

Values, expressed in the units indicated in column 1, are the means for groups of eight pigs, and that marked with an asterisk is significantly ($P < 0·05$) greater than the corresponding values for diets 1 and 3.

Liver composition

The proximate composition of the liver also failed to demonstrate any statistically significant effects of treatment (Table 6). The percentage of water (81·0%) was greater and the percentages of fat (3·1%) and protein (14·7%) were lower in the livers of comparably aged sow-reared pigs than in the livers of pigs fed the starch-containing diets.

Conclusion

Under the conditions of this study, it thus appears that over a 25-day period, growth,

Table 6. *Effect of carbohydrate source on liver composition of miniature pigs*

Component	Liver content (%) in pigs fed diet no.				Standard error of the mean
	1	2	3	4	
Liver weight (g)...	139	125	132	125	11·1
(% of body weight)...	4·04	4·02	4·00	3·85	0·185
Wet liver					
Water	75·1	73·5	74·1	73·8	0·41
Fat	5·7	6·1	5·5	5·1	0·38
Protein	18·2	18·4	18·6	19·8	0·55
Ash	1·4	1·4	1·4	1·4	0·03
Fat-free wet liver					
Water	79·6	78·1	78·4	77·8	0·44
Protein	19·3	19·6	19·7	20·8	0·57
Ash	1·5	1·5	1·5	1·5	0·03

Values are means for groups of eight pigs.

blood chemistry and the composition of the liver and carcass were similar whether young Pitman–Moore miniature pigs were fed a "thin-boiling" waxy corn starch or various chemical modifications of this starch, prepared as described in Sec. 121.1031(a,d,e) of Title 21 of the Code of Federal Regulations.

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REFERENCES

Anderson, T. A. & Fomon, S. J. (1971). Commercially prepared strained and junior foods for infants. Nutritional considerations. *J. Am. diet. Ass.* **58,** 520.

Bowland, J. P. (1966). Swine milk composition—a summary. In *Swine in Biomedical Research.* Edited by L. K. Bustad and R. O. McClellan. p. 97. Frayn Printing Co., Seattle.

Bustad, L. K. & McClellan, R. O. (1966). *Swine in Biomedical Research.* Proceedings of a Symposium at the Pacific Northwest Laboratory, Richland, Washington, July 19–22, 1965. Frayn Printing Co., Seattle.

Duncan, D. B. (1955). Multiple range and multiple F tests. *Biometrics* **11,** 1.

Elliott, R. F., Vander Noot, G. W., Gilbreath, R. L. & Fisher, H. (1971). Effect of dietary protein level on composition changes in sow colostrum and milk. *J. Anim. Sci.* **32,** 1128.

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

Filer, L. J., Jr., Fomon, S. J., Anderson, T. A., Andersen, D. W., Rogers, R. R. & Jensen, R. L. (1973). Growth, serum chemical values and carcass composition of Pitman–Moore miniature pigs during the first eight weeks of life. *J. Nutr.* **103,** 425.

Fomon, S. J. (1967). *Infant Nutrition.* p. 93. W. B. Saunders Company, Philadelphia.

Joint FAO/WHO Expert Committee on Food Additives (1972). Toxicological Evaluation of Some Enzymes, Modified Starches and Certain Other Substances. *F.A.O. Nutr. Mtg Rep. Ser. no.* 50, p. 23; *Tech. Rep. Ser. Wld Hlth Org.* **488,** p. 23.

Schneider, D. L. & Sarett, H. P. (1966). Use of the hysterectomy-obtained SPF pig for nutritional studies of the neonate. *J. Nutr.* **89,** 43.

Snedecor, G. W. (1956). *Statistical Methods Applied to Experiments in Agriculture and Biology.* 5th ed. p. 237. Iowa State University Press, Ames, Iowa.

Subcommittee on Swine Nutrition, Committee on Animal Nutrition, Agricultural Board and National Research Council (1968). *Nutrient Requirements of Swine.* 6th revised ed. p. 1. National Academy of Sciences Publ. no. 1599, Washington.

Sutherland, T. M. (1965). The correlation between feed efficiency and rate of gain, a ratio and its denominator. *Biometrics* **21,** 739.

Wurzburg, O. B. & Szymanski, C. D. (1970). Modified starches for the food industry. *J. agric. Fd Chem.* **18,** 997.

Effet de modifications de l'amidon de maïs cireux sur la croissance, les caractéristiques biochimiques du sérum et la composition des tissus chez le porc miniature Pitman–Moore

Résumé—On a sevré à l'âge de 3 jours quatre groupes de chacun huit porcs miniatures Pitman–Moore, puis on les a nourris pendant 25 jours de rations identiques, au type d'hydrate de carbone près. Une formule contenait de l'amidon de maïs cireux à ébullition fine et les 3 autres des modifications chimiques différentes de cet amidon (phosphate d'amylopectine phosphatée, phosphate d'amylopectine et glycérolé d'amylopectine hydroxypropylée). On n'a observé aucun effet statistiquement significatif et en corrélation avec le traitement sur la croissance, les caractéristiques biochimiques du sang ou du sérum et la composition de la carcasse ou du foie.

Der Einfluss von "Waxy-Corn"-Stärke-Modifikation auf das Wachstum, die biochemischen Serumwerte und die Körperzusammensetzung von Pitman-Moore-Zwergschweinen

Zusammenfassung—Vier Gruppen von je acht Pitman–Moore-Zwergschweinen wurden im Alter von 3 Tagen abgesetzt und dann 25 Tage lang künstlich ernährt, und zwar gleichartig mit Ausnahme des Kohlehydrattyps. Das Futter enthielt dünnkochende "Waxy-Corn"-Stärke oder eine von drei chemischen Modifikationen dieser Stärke (phosphatiertes Distärkephosphat, Distärkephosphat und hydroxypropyliertes Distärkeglycerol). Es wurden keine statistisch signifikanten, mit dieser Art der Fütterung in Beziehung stehenden Wirkungen auf das Wachstum, die biochemischen Blut- oder Serumwerte oder die Körper oder Leberzusammensetzungen beobachtet.

Fd Cosmet. Toxicol. Vol. 11, pp. 755–769. Pergamon Press 1973. Printed in Great Britain

Long-term Feeding and Reproduction Studies on Emulsifier YN in Rats

P. G. BRANTOM and I. F. GAUNT—*Toxicology*
JOAN HARDY and P. GRASSO—*Pathology*
and
S. D. GANGOLLI—*Biological Chemistry*
British Industrial Biological Research Association, Woodmansterne Road, Carshalton, Surrey, England

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Abstract—Emulsifier YN was fed to groups of 48 male and 48 female rats at dietary levels of 0 (control), 2 or 6% for 2 yr. A similar group of rats was fed on diet containing 4% soya lecithin. These treatments did not adversely affect mortality, rate of body-weight gain, haematology, urine constituents, renal concentrating ability, serum constituents or the incidence of tumours. Thyroid weight was increased in all treated groups, but this was the result of an increased incidence of parathyroid hyperplasia, thought to be due to spontaneous renal changes combined with an elevated intake of phosphate. A slightly increased incidence of myocardial fibrosis was also associated with the parathyroid hyperplasia. The incidence and severity of other histopathological changes were not influenced by feeding YN or lecithin.

A two-generation study, in which the parent animals were fed diets containing 0 or 6% YN for 13 wk before mating, revealed no effects on fertility, the number of young or the growth of young during lactation, and no abnormalities were seen.

The no-untoward-effect level for YN in this study was 6%, approximately equivalent to an intake by the rat of 3 g/kg/day.

INTRODUCTION

Emulsifier YN (referred to subsequently as YN) is used as an emulsifier and viscosity-reducing agent in the manufacture of chocolate. Previously, natural lecithins were used for this purpose, but the only one available in sufficient quantity for commercial use was derived from soya beans and had too strong a flavour for use in some types of chocolate. YN was developed as a substitute and is used in chocolate at levels of 0·35–0·5%.

YN consists of a mixture of ammonium salts of phosphatidic acids and triglycerides derived from rapeseed oil. It is produced by phosphorylation of the products of glycerolysis of partially-hardened rapeseed oil with final neutralization of the reaction mixture with ammonia. Six phosphatidic acids have been identified in the mixture before neutralization; these are mono-, bis- and tris-phosphatidic acids, lysophosphatidic acid (or equivalent 1,2-cyclo form), bis-phosphatidyl-lysophosphatidic acid (or equivalent 1,2-cyclo form) and bis-phosphatidyl-monophosphatidic acid. It is noteworthy that the relative contribution of each of these components to the mixture is not known and that the percentage composition probably varies with different batches.

A. C. Frazer (unpublished report 1954) demonstrated, in rats, that YN was absorbed in

the presence of fats without impairing fat absorption or other intestinal functions. No effects on survival, growth, liver and kidney function or histological appearance of the organs were observed in rats fed diets containing 1% YN or 26% chocolate containing 5% YN for a period in excess of 45 wk. A. C. Frazer (unpublished report 1954) reported also that rats, rabbits and dogs survived a single oral dose of 5 g YN/kg, and that rats and guinea-pigs survived an intramuscular dose of 2 g YN/kg. Acute studies in rats, mice and rabbits showed that the LD_{50} values were in excess of 20 g/kg orally and 10 g/kg after intraperitoneal injection (J. Colley and I. F. Gaunt, unpublished data 1967).

Feuer (1967) compared the *in vivo* absorption and distribution of ^{32}P-labelled YN and inorganic phosphate in rats. Approximately 80% of the radioactivity associated originally with the YN was excreted in the faeces and, from *in vitro* studies, it was concluded that the remainder was degraded in the gastro-intestinal tract, liberating inorganic phosphate which was distributed and excreted as part of the phosphate pool of the body.

Twice-weekly intraperitoneal injections of 2 g YN/kg for 5 wk produced no deleterious effects on growth, relative spleen weight, haematology or corpuscular fragility (J. Colley and I. F. Gaunt, unpublished data 1967). Gaunt, Grasso & Gangolli (1967) found no ill effects in groups of 15 male or 15 female rats fed YN at 0, 0·75, 1·5, 3·0 or 6·0% of the diet for 90 days. Rats fed 6·0% soya lecithin in the diet for 90 days showed a slight transient anaemia.

YN was considered by the Joint FAO/WHO Expert Committee on Food Additives (1970), which suggested that it should be temporarily accepted for use in food provided that long-term data in the rat were made available by 1974. At the time of the suggestion such a study was already in progress at BIBRA as part of the safety evaluation programme and the results are reported in this paper.

EXPERIMENTAL

Materials. YN was supplied by Cadbury Brothers Ltd., Bourneville, Birmingham, England, and conformed to the manufacturer's specification for YN for use in foodstuffs. It was described as consisting of a mixture of ammonium compounds of phosphatidic acids derived from rapeseed oil, and a proportion of triglycerides from the partially-hardened oil. The impurities did not exceed 0·2% water, 2·5% matter insoluble in 40–60 petroleum ether, 0·2% inorganic matter insoluble in 40–60 petroleum ether, 42% unreacted triglyceride, 3·0–3·4% phosphorus, 2·5 ppm arsenic, 2 ppm lead and 2 ppm copper; the pH of the mixture was between 6 and 8. The soya-bean lecithin used in this study was also supplied by Cadbury Brothers.

Animals and diet. Both sexes of Wistar rats, obtained from an SPF colony, were maintained on reground Spillers' Laboratory Small Animal Diet and water *ad lib.* They were housed in a room kept at a temperature of 21 ± 1°C with a relative humidity of 50–60%.

Experimental design and conduct

Long-term feeding study. Groups of 48 male (100–130 g) and 48 female (90–120 g) weanling rats caged in groups of four were fed diets containing either 0 (control), 2 or 6% YN or 4% soya lecithin for 2 yr.

The general condition and behaviour of the animals were observed and any animal found to be in ill health was isolated, to be returned to its cage on recovery or to be killed when moribund. Body weights and food consumption were recorded at intervals up to wk 95.

Body weights were also recorded at wk 102 and at autopsy. Blood for haematological examination was collected at 13, 26, 54 and 81 wk from the caudal veins of ten rats of each sex, from the groups fed control diet, 6% YN and 4% soya lecithin. At 102–106 wk blood was collected from the aorta of all surviving rats while they were under barbiturate anaesthesia prior to autopsy. All the blood samples were examined for haemoglobin concentration and counts were made of the different types of leucocytes. Additionally, at 13, 26 and 54 wk all samples were examined for packed cell volume and counts were made of red blood cells, total leucocytes and reticulocytes.

Serum was separated from blood taken at autopsy and analysed for the content of urea, glucose, total protein and albumin and for the activities of glutamic–oxalacetic and glutamic–pyruvic transaminases and lactic dehydrogenase.

Urine analyses and kidney-function tests were carried out at wk 13, 26 and 52, on ten animals of each sex from the control group and the group fed 6% YN. The urine was examined for appearance, microscopic constituents and content of glucose, bile salts, ketones and blood. The renal concentrating ability was assessed by measuring the specific gravity and volume of the urine produced during a 6-hr period of water deprivation and of that produced during a 4-hr period commencing 16 hr after an oral water load of 25 ml/kg. To assess the renal diluting ability, the volume and specific gravity of the urine produced in the first 2 hr after the water load were also measured. Urinary cell excretion was estimated by counting the number of cells in the urine collected in the first 2 hr after the water load.

Autopsies were carried out on all rats killed because of ill health and on any that died, unless this was precluded by cannibalism or advanced autolysis. At 2 yr, all remaining rats were killed by exsanguination under barbiturate anaesthesia and autopsies were performed. The animals were examined for macroscopic abnormalities and the brain, pituitary, thyroid, heart, liver, spleen, stomach, small intestine, caecum, kidneys, adrenal glands and gonads were weighed. Samples of these organs and samples of salivary gland, trachea, lung, aortic arch, skeletal muscle, lymph nodes, colon, rectum, pancreas, spinal cord, bone and uterus and any other tissue that appeared abnormal were preserved in 10% buffered formalin. All tissues from control animals and those fed 4% soya lecithin or 6% YN together with all macroscopically abnormal tissues from the group fed 2% YN were embedded in paraffin wax and sections were stained with haematoxylin and eosin for microscopic examination.

Reproduction study. Groups of ten male and 30 female weanling rats (F_0 generation) were fed 0 (control) or 6% YN for 13 wk, after which each group was divided into sub-groups of one male and three females, which were caged together for up to 21 days. The date of mating was established by the observation of spermatozoa in vaginal smears. All females were caged individually when obviously pregnant or after 21 days with the male.

The females were allowed to give birth to their litters (F_{1a} generation). As soon as possible after birth all these litters were weighed and the sex ratio and any obvious abnormalities were recorded. The young from 20 of the females from each group were reared to 21 days while those from the remaining ten in each group were autopsied one day after birth. A 4, 14 and 21 days after birth the total weights of pups of each sex were recorded for all litters surviving to these times. After 21 days the remaining litters were killed and each pup was examined for macroscopic abnormalities.

One week after the weaning of the last litter, the rats were remated, in the sub-groups described above. All litters from this mating (F_{1b} generation) were allowed to survive until 21 days after birth and the observations were carried out as described for the F_{1a} generation. At 21 days the pups were marked according to parentage and all the offspring at each treat-

ment level were grouped according to sex. Thirty females and ten males were selected at random from the offspring of each group and fed on the same diet as their parents for 13 wk. The remaining pups were then killed and examined for gross abnormalities.

After the 13-wk treatment, the selected animals from the F_{1b} generation were mated as for the F_0 generation animals, with observations and recordings on the litters (F_{2a} generation) as for the F_{1a} generation. The selected F_{1b} animals were then remated. Seven control and eight YN-treated females were killed on day 18 of pregnancy. Counts were made of the numbers of implantations, resorptions, live pups and corpora lutea. The position of each pup in the uterus was noted and all were examined for external abnormalities. Alternate pups were preserved for possible alizarin staining and the remainder were dissected under a low-power microscope and examined for visceral abnormalities. The remaining 20 females of each group were allowed to give birth to their litters (F_{2b} generation) which were observed as for the F_{1a} generation. As no differences between treated and control animals were demonstrated by this two-generation study, further matings were not considered necessary.

RESULTS

Long-term study

No abnormalities were seen in the behaviour of the rats. Approximately 42% of the males and 32% of the females died or were killed *in extremis* during the study, but there was no significant difference between the groups in the number of animals dead at any particular time (Table 1).

Table 1. *Cumulative death record of rats fed diets containing 0, 2 or 6% YN or 4% soya lecithin (SL) for 2 yr*

	Total no. of deaths in							
	Males fed dietary levels (%) of				Females fed dietary levels (%) of			
Time (wk)	0	4 (SL)	2 (YN)	6 (YN)	0	4 (SL)	2 (YN)	6 (YN)
Up to 40	0	1	0	0	0	1	0	0
48	0	1	1	0	0	1	0	0
56	1	1	1	1	1	1	1	0
64	1	1	1	3	2	2	1	0
72	2	2	2	5	2	4	2	0
80	8	7	4	6	5	5	4	4
88	10	14	9	10	6	7	5	5
96	16	19	17	16	7	12	9	14
100	22	22	22	19	9	14	12	18

The values are the numbers of rats dead or killed *in extremis* from groups of 48 animals.
Numbers of deaths in groups of treated animals did not differ significantly (Chi-squared) from those in control groups.

Body-weight gain in all treated groups was greater than in controls (Table 2) and, on occasions from wk 42 onwards, the differences were statistically significant in the case of the males fed 2% YN. Similarly, the body weights of females fed 6% YN were significantly higher than those of controls from wk 62 onwards. These values did not differ significantly from those of the group fed 4% soya lecithin. The food intakes of all treated male groups

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Table 2. *Mean body weight, food consumption and emulsifier intake of rats fed diets containing 0, 2 or 6% YN or 4% soya lecithin (SL) for 2 yr*

Dietary level (%)	Body weight (g) at wk				Food consumption (g/rat/day) at wk				Mean food consumption (g/rat/day)	Emulsifier intake† (g/kg/day) at wk				Mean intake (g/kg/day)	Calculated intake (kg) by wk 95
	0‡	29	56	95	0‡	29	56	95		0‡	29	56	95		
Males															
0	132	541	659	680	18·7	22·9	23·1	21·1	21·2	—	—	—	—	—	—
4 (SL)	129	565	671	711	19·3	22·0	25·4	24·2	22·7	5·68	1·56	1·51	1·36	1·47	0·6
2 (YN)	130	569	698	727	19·1	20·1	27·5	20·3	22·6	2·81	0·71	0·79	0·56	0·68	0·3
6 (YN)	131	569	689	717	19·7	21·7	27·7	22·7	22·9	8·58	2·29	2·41	1·90	2·32	0·9
Females															
0	114	337	369	444	16·1	19·3	21·1	22·2	19·4	—	—	—	—	—	—
4 (SL)	118	312	384	452	17·1	19·0	22·6	24·5	20·7	5·57	2·43	2·36	2·17	2·28	0·6
2 (YN)	118	308	382	470	17·0	18·9	21·8	20·7	19·3	2·76	1·23	1·14	0·88	1·08	0·3
6 (YN)	118	308	385	492*	16·7	18·4	21·9	20·5	19·3	8·23	3·58	3·42	2·50	2·89	0·8

†Calculated from data on body weight and food consumption.
‡First day of feeding.
The values shown for body weight are means for all survivors in each group. That marked with an asterisk differs significantly (Student's *t* test) from the control value: $*P < 0{\cdot}05$.
The values for food consumption represent the means for 12 cages of four rats.

were slightly higher than of controls, whereas females from treated and control groups consumed similar amounts of food daily (Table 2). The calculated daily and total intakes of both YN and soya lecithin during the study are shown in Table 2.

There were no statistically significant differences between treated and control animals in the results of serum analyses (Table 3) or the tests of renal concentrating ability (Table 4). The results of the haematological investigations did not reveal any significant differences between treated and control animals. Table 5 shows the results for wk 52 and 102.

Table 3. *Results of serum analyses of rats fed diets containing 0, 2 or 6% YN or 4% soya lecithin (SL) for 2 yr*

Dietary level (%)	No. of rats	GOT (IU)	GPT (IU)	LDH (IU)	Glucose (mg/100 ml)	Urea (mg/100 ml)	Total protein (g/100 ml)	Albumin (g/100 ml)
				Males				
0	24	44	8	1039	139	34	6·[illegible]	3·3
4 (SL)	25	43	8	976	121	34	6·[illegible]	3·3
2 (YN)	24	46	8	1020	124	33	6·	2·9
6 (YN)	25	44	8	1067	134	36	6·[illegible]	3·0
				Females				
0	37	47	9	881	119	33	7·[illegible]	3·3
4 (SL)	32	45	7	954	127	34	7·[illegible]	3·3
2 (YN)	33	45	10	912	134	31	8·	3·7
6 (YN)	26	43	8	998	130	31	8·[illegible]	4·0

GOT = Glutamic–oxalacetic transaminase GPT = Glutamic–pyruvic transaminase
LDH = Lactic dehydrogenase
Values are means for the numbers of rats shown.

The only organ-weight changes found were increases in the thyroid (Table 6) and these were apparent in all treated groups. These increases varied from 17 to 27% in males and from 11 to 26% in females. At autopsy it was noted, mainly in males, that small nodules were present on the surface of the thyroids of four controls and seven or eight animals in each treated group, and histological examination of these tissues revealed enlarged hyperplastic parathyroids (Table 7). This lesion was also found in rats in which no nodules were seen at autopsy.

It was also noted at autopsy that the kidneys of most rats were enlarged and brown coloured and had cortical pits and cysts. Histological examination revealed changes described as glomerulonephrosis in most animals. The severity of this lesion varied considerably but, in general, was much more severe in the males. Myocardial fibrosis was found in almost half the rats in all groups including controls. In females the incidence was similar in treated and control rats but in males the incidence of this finding was increased in the treated rats.

There was a variety of other histopathological changes (Table 7) but the severity and incidence of these were similar in all groups, including the controls.

The incidence of tumours seen in this study is summarized in Table 8 the commonest being chromophobe adenoma of the pituitary and fibroadenoma of the mammary tissue. Benign tumours affecting the liver, pancreas, pituitary, thyroid, adrenals, testes, skin, brain,

Table 4. *Results of renal concentration/dilution test and urinary cell excretion of rats fed diets containing 0 or 6% YN for 13, 26 and 52 wk*

Sex and dietary level (%)	No. of rats examined	Cell excretion (10^3/hr)	Concentration test				Dilution test (0–2 hr)	
			Specific gravity		Volume (ml)			
			0–6 hr	16–20 hr	0–6 hr	16–20 hr	Specific gravity	Volume (ml)
			Wk 13					
Male								
0	10	2·8	1·067	1·072	1·5	1·2	1·013	6·5
6 (YN)	10	2·2	1·068	1·067	1·2	1·0	1·021	5·0
Female								
0	10	2·0	1·042	1·083	1·2	0·4	1·005	6·1
6 (YN)	10	2·4	1·054	1·072	0·9	0·7	1·004	5·6
			Wk 26					
Male								
0	10	4·9	1·060	1·066	1·5	1·8	1·006	9·3
6 (YN)	10	4·0	1·052	1·062	2·1	2·1	1·014	6·9
Female								
0	10	3·9	1·039	1·074	1·4	0·3	1·004	5·6
6 (YN)	10	3·3	1·055	1·073	0·8	0·6	1·006	5·5
			Wk 52					
Male								
0	10	1·5	1·034	1·054	2·9	1·9	1·000	10·4
6 (YN)	10	1·6	1·040	1·053	1·4	2·0	1·011	7·6
Female								
0	10	2·0	1·035	1·059	2·3	0·1	1·006	5·6
6 (YN)	10	2·8	1·039	1·074	1·6	0·8	1·007	6·7

Figures are means for the numbers of rats shown.
Tests for blood, bile salts and reducing substances were negative in all groups.

salivary gland, ovary, uterus, prostate and connective tissue were also found. Malignant tumours were found in all groups (Table 8), affecting the pancreas, thymus, salivary gland, mammary tissue, uterus, skin and connective tissue. However, the incidence of tumours was not influenced by the feeding of YN or soya lecithin.

Reproduction study

The data on the two generations of rats reared to weaning are shown in Table 9. YN treatment had no effect on fertility, the number of young born, the number reared or the weight gain of the young. The sex ratio of the young was also noted and in all cases this was unaffected by treatment and was approximately 1:1. Autopsy of the young at weaning age revealed no abnormalities. The observations made on the litters of selected females killed during the second pregnancy of the second generation are shown in Table 10. This study showed that treatment with 6% YN for two generations did not adversely affect implantation or subsequent foetal development. Only one abnormal foetus, with exencephalus, was found. This was from a control litter.

Table 5. *Results of haematological investigations in rats fed diets containing 0 (control), 2 or 6% YN or 4% soya lecithin (SL) for 54 or 102 wk*

Sex and dietary level (%)	No. of rats	Hb (g/100 ml)	PCV (%)	RBC (10^6/ mm^3)	Retics (% of RBC)	Leucocytes: Total (10^3/ mm^3)	Leucocytes: Differential (%) N	E	L	M
Wk 54										
Male										
0	10	16·2	49	7·63	0·67	11·5	23	2	74	1
4 (SL)	10	16·6	49	7·76	0·70	10·3	18	2	79	1
6 (YN)	10	16·4	49	7·69	1·04	10·3	18	2	79	1
Female										
0	10	15·4	48	6·37	0·46	7·5	21	2	76	1
4 (SL)	10	15·6	49	5·81	0·87	7·8	18	2	78	2
6 (YN)	10	14·9	47	5·61	0·74	6·1	19	3	77	1
Wk 102										
Male										
0	25	13·9	—	—	—	—	40	1	57	2
4 (SL)	25	14·0	—	—	—	—	40	2	57	1
2 (YN)	24	13·9	—	—	—	—	—	—	—	—
6 (YN)	26	14·3	—	—	—	—	38	1	59	2
Female										
0	39	14·4	—	—	—	—	43	1	54	2
4 (SL)	31	14·1	—	—	—	—	42	1	56	1
2 (YN)	35	13·9	—	—	—	—	—	—	—	—
6 (YN)	29	14·0	—	—	—	—	42	1	57	2

Hb = Haemoglobin PCV = Packed cell volume RBC = Red blood cells Retic = Reticulocytes
N = Neutrophils E = Eosinophils L = Lymphocytes M = Monocytes
Basophils did not constitute more than 0·5% of the leucocytes in any group.

DISCUSSION

This study has shown that the feeding of YN to rats for 2 yr at dietary levels up to 6% does not adversely affect survival, renal function, haematological parameters or serum chemistry. In addition, the reproductive performance of the rats was not affected and no signs of foetal toxicity or teratogenicity were encountered. These observations confirm the lack of toxic effect found by Gaunt *et al.* (1967) in a short-term feeding study in rats utilizing the same dose levels as those employed in the present work. It seems likely that the increased body-weight gain seen in all treated groups, including those fed 4% soya lecithin, was a result of the increased consumption of lipid rather than of any specific effect of YN.

Slight increases in thyroid weight in all the treated groups, particularly the males, were not associated with any histopathological changes in this organ. However, it was noticed that the parathyroid glands were increased in size more frequently in treated than in control animals and this observation was confirmed by the histological finding of parathyroid hyperplasia. This effect was also more noticeable in the male animals and, in general, glomerulonephrosis was also more severe in this sex.

Snell (1967) summarized earlier work showing that prolonged renal damage can lead to parathyroid hyperplasia in the rat. It is postulated (Snell, 1967) that failure of the kidney to excrete phosphate causes an imbalance in the calcium-phosphorus ratio and the activity of

Table 6. *Organ weights of rats fed diets containing 0, 2 or 6% YN or 4% soya lecithin (SL) for 2 yr*

Dietary level (%)	No. of rats examined	Organ weights												Terminal body weight (g)
		Brain	Heart	Liver	Spleen	Kidneys	Stomach	Small intestine	Caecum	Adrenals†	Gonads‡	Pituitary†	Thyroid†	
Absolute organ weights (g)														
Males														
0	25	2·18	1·52	15·44	1·43	4·48	2·72	9·57	1·44	81	3·32	14	36	622
4 (SL)	25	2·22	1·58	16·14	1·67	4·52	2·84	10·27	1·54	77	3·23	13	46	670
2 (YN)	24	2·14	1·64	16·15	1·71	4·61	2·82	9·83	1·53	88	3·29	13	43	633
6 (YN)	25	2·21	1·64	15·37	1·39	4·40	2·71	9·85	1·54	99	3·79	13	45	655
Females														
0	39	1·95	1·15	11·66	1·06	2·78	2·06	7·90	1·21	73	101	17	27	423
4 (SL)	31	1·99	1·21	11·66	1·07	2·71	2·03	7·87	1·25	78	122	16	30	422
2 (YN)	35	1·97	1·22	11·85	1·15	2·55	2·11	7·98	1·28	77	115	15	31	448
6 (YN)	29	1·97	1·27	11·60	1·11	2·63	2·16	7·97	1·27	71	109	19	34**	465
Relative organ weights (g/100 g body weight)														
Males														
0	25	0·35	0·24	2·49	0·23	0·72	0·64	1·54	0·23	13	0·53	2·2	6·0	—
4 (SL)	25	0·33	0·24	2·41	0·25	0·68	0·42	1·53	0·23	12	0·48	2·0	7·3	—
2 (YN)	24	0·34	0·26	2·55	0·27	0·68	0·45	1·55	0·24	14	0·52	2·2	6·9	—
6 (YN)	25	0·34	0·25	2·33	0·21	0·67	0·41	1·49	0·24	15	0·53	2·0	6·8	—
Females														
0	39	0·46	0·27	2·76	0·25	0·66	0·49	1·87	0·29	17	24	4·1	7·0	—
4 (SL)	31	0·47	0·29	2·77	0·25	0·64	0·48	1·87	0·30	19	29	3·7	7·1	—
2 (YN)	35	0·44	0·27	2·65	0·26	0·57	0·47	1·78	0·29	17	26	3·4	7·2	—
6 (YN)	29	0·42	0·27	2·49	0·24	0·58	0·47	1·71	0·27	15	23	4·0	7·5	—

†Weights of this organ are expressed in mg and mg/100 g body weight.
‡Values for the weights of ovaries are expressed in mg and mg/100 body weight.
Values are means for the numbers of animals shown and that marked with asterisks differs significantly (Student's *t* test) from the control value: $^{**}P < 0{\cdot}01$.

Table 7. *Incidence of histological findings (excluding tumours) in rats fed diets containing 0–6% YN or 4% soya lecithin (SL)*

Tissue and type of abnormality	No. of animals affected among							
	Males fed dietary levels (%) of				Females fed dietary levels (%) of			
	0	4 (SL)	2 (YN)	6 (YN)	0	4 (SL)	2 (YN)	6 (YN)
No. of rats examined...	41	39	39	39	47	47	45	44
Kidneys								
Glomerulonephrosis	41	39	39	39	47	45	41	44
Heart and blood vessels								
Myocardial fibrosis	15	19	20	24	15	19	—	11
Calcification	5	3	5	1	0	2	—	0
Medial hypertrophy	1	2	1	0	5	1	—	2
Liver								
Nodules	1	1	0	0	1	0	0	1
Bile-duct proliferation	13	14	15	17	24	24	20	27
Foci of fatty degeneration	12	7	13	9	8	13	16	12
Generalized fatty change	1	4	2	3	4	7	0	5
Stomach and intestine								
Ulceration of mucosa	4	1	2	1	0	0	0	0
Submucosal inflammation	2	1	2	2	1	0	1	0
Parathyroids								
Hyperplasia	9	16	15	20	3	4	—	7
Thyroid								
Large colloid follicles	1	2	0	0	2	1	1	2
Pituitary								
General hyperplasia	1	1	5	4	9	8	7	3
Haemorrhage	4	5	2	9	12	1	1	2
Adrenals								
Cortical-cell degeneration	19	7	6	13	16	20	—	20
Haemorrhage	2	0	5	0	18	14	—	12
Thymus								
Haemorrhage	2	4	1	0	0	0	0	0
Lungs								
Alveoli with foamy macrophages	1	7	6	4	7	14	—	13
Leucocyte infiltration	7	8	5	10	2	6	—	6
Congestion	11	4	9	9	1	1	—	1
Testes								
Partial atrophy	6	2	3	1	—	—	—	—
Ovaries								
Cystic	—	—	—	—	11	6	9	17
Atrophy	—	—	—	—	2	0	1	0
Uterus								
Cystic	—	—	—	—	3	4	—	2
Endometritis	—	—	—	—	1	5	—	1
Hydrometra	—	—	—	—	0	4	—	2

The figures indicate the incidence of the finding in the number of rats shown. A dash indicates that the organ was not examined.

Table 8. *Incidence of tumours in rats fed dietary levels of 0, 2 or 6% YN or 4% soya lecithin (SL)*

Organ and type of tumour	No. of animals with tumours among							
	Males fed dietary levels (%) of				Females fed dietary levels (%) of			
	0	4 (SL)	2 (YN)	6 (YN)	0	4 (SL)	2 (YN)	6 (YN)
No. of rats examined...	41	39	39	39	47	47	45	44
Liver								
Cholangioma	0	1	1	0	0	0	0	0
Small intestine								
Adenoma	0	0	0	1	0	0	0	0
Pancreas								
Islet-cell tumour	1	0	1	0	0	0	0	1
Adenoma (exocrine)	1	0	0	3	0	0	0	0
Adenocarcinoma	0	0	0	1	0	0	0	0
Pituitary								
Chromophobe adenoma	3	3	1	2	13	13	9	23
Thyroid								
Adenoma	0	0	0	1	3	0	0	0
Adrenals								
Haemangioma	0	0	0	1	0	0	0	0
Phaechromocytoma	0	0	0	0	0	0	1	0
Cortical tumour	0	0	0	0	1	0	0	0
Thymus								
Lymphosarcoma	0	1	0	1	0	0	1	1
Thymoma	0	0	1	0	1	1	1	1
Testes								
Interstitial-cell adenoma	1	1	0	1	—	—	—	—
Skin								
Keratoacanthoma	1	1	0	3	0	0	0	0
Squamous carcinoma	0	0	0	2	0	1	0	1
Connective tissue								
Lipoma	4	1	0	1	2	6	1	2
Fibroma	2	0	0	2	2	1	0	0
Fibrosarcoma	3	1	1	0	0	0	0	0
Brain								
Glioma	1	0	0	0	0	0	0	0
Salivary gland								
Adenoma	0	0	0	1	0	0	0	0
Adenocarcinoma	0	0	0	1	0	0	0	0
Mammary tissue								
Fibroadenoma	0	0	0	0	19	11	12	6
Adenocarcinoma	0	0	0	0	1	3	1	3
Ovary								
Theca cell tumour	—	—	—	—	0	0	1	0
Uterus								
Fibromyoma	—	—	—	—	0	1	2	0
Adenocarcinoma	—	—	—	—	0	1	0	0
Fibrosarcoma	—	—	—	—	0	0	0	1
Prostate								
Cystadenoma	1	0	0	0	—	—	—	—

Table 9. *Results of reproduction study in rats fed 0 or 6% YN in the diet*

Dietary level (%)	No. in litter at day				Total weight of litter (g) at day				Mean pup weight (g) at day				No. pregnant/no. mated	No. fertile males	No. of litters born
	B	4	14	21	B	4	14	21	B	4	14	21			
First generation (F_1)															
First litter (F_{1a})															
0	11·4	10·8	10·4	10·4	64·5	100·0	304·0	463·1	5·7	9·5	29·7	44·9	27/30	10/10	27†
6	10·5	9·1	9·1	9·1	62·8	96·9	301·9	475·3	5·4	9·7	29·8	46·9	30/30	10/10	30‡
Second litter (F_{1b})															
0	12·5	11·7	11·7	11·2	75·4	101·6	331·1	533·6	5·3	11·3	29·8	49·9	30/30	10/10	29
6	11·4	11·4	11·1	11·1	72·6	120·5	348·1	585·3	6·2	10·6	27·0	47·8	29/29	10/10	27
Second generation (F_2)															
First litter (F_{2a})															
0	11·2	10·9	10·8	10·8	70·7	94·7	305·2	460·4	6·2	8·7	28·7	45·4	28/30	10/10	28
6	11·2	10·6	10·5	10·5	71·8	91·8	312·8	480·4	6·2	8·8	28·7	45·3	27/30	10/10	27
Second litter (F_{2b})															
0	10·0	10·0	10·0	10·0	57·1	100·4	252·6	417·1	6·6	10·0	26·2	42·5	24/30	10/10	17*
6	11·3	11·1	10·5	10·5	65·7	101·7	267·8	457·6	6·0	9·3	24·8	43·6	27/30	10/10	19*

B = Day the litter was born

*Seven control and eight test females were killed before parturition for *in utero* examination of pups.

†One litter died as the mother was accidentally killed.

‡Two litters killed by mother within 11 days of birth.

Table 10. In utero *observations on the second litters produced by rats of the F_2 generation fed 0 or 6% YN in the diet*

Dietary level (%)	No. of litters	No. of resorptions/ pregnant female	No. of dead pups/ pregnant female	No. of live pups/ pregnant female	Total no. of implanta-tions/ pregnant female	No. of corpora lutea/ pregnant female	Abnorm-alities seen in pups
0	7	0·57	0·28	9·1	10·0	13·4	Exencephalus in 1 pup
6	8	0·63	0	11·8	12·4	15·3	None

These observations were made on the litters of females killed 2 or 3 days before parturition.

the parathyroids is stimulated in an attempt to redress the balance. In the present study, it was noticed that parathyroid hyperplasia occurred in animals with severe renal damage. It would appear that the parathyroid hyperplasia in controls was due to long-standing renal damage.

However, in the present study, renal lesions were no more severe in the treated animals than in the controls, so the increased incidence of parathyroid hyperplasia in the treated rats could not be attributed solely to renal damage. It is known (Feuer, 1967) that the phosphorus in YN contributes to the normal phosphate pool and so represents an increased intake of phosphate. In the absence of renal damage this increased intake of phosphate from YN does not itself lead to parathyroid hyperplasia, since in a 3-month study in which YN was fed to rats at levels up to 6%, Gaunt *et al.* (1967) found no evidence of this effect. Nevertheless, the greater incidence of parathyroid hyperplasia in the treated rats probably results from the imposition of an increased intake of phosphate on rats with spontaneous renal damage, and as such is not likely to be relevant to the assessment of the toxic hazard of YN in man.

Lehr (1959 & 1965) showed that cardiac necrosis occurred in rats with parathyroid hyperplasia associated with renal insufficiency, and that this effect could be abolished by prior removal of the parathyroids. It seems possible, therefore, that the fibrosis seen in the myocardium of the rats of all groups in this study was a consequence of the parathyroid hyperplasia discussed above and, moreover, that the slightly increased incidences found with the treated animals were not a direct result of the feeding of YN or soya lecithin.

Many of the tumours found in the treated rats in this study also occurred with a similar incidence in untreated rats and so cannot be attributed to either YN or soya-lecithin treatment. One tumour, an adenocarcinoma of the uterus occurred only in one rat fed on the diet containing lecithin, but this type of tumour has been shown to occur with a frequency of 1–2% in various strains of rat (Snell, 1965).

With regard to the tumours occurring in YN-treated rats but not in the controls, four (cholangioma, adrenal phaeochromocytoma, ovarian theca cell tumour and uterine fibromyomas) were not found in the rats given the highest dietary level of YN. All of these tumours have been reported in untreated rats. Cholangiomas were described in the livers of untreated Wistar rats (Lemon, 1967) and a similar lesion was described as a cystadenoma by Bullock & Curtis (1930). Phaeochromocytomas were described as a common finding in a colony of Wistar rats (Gilbert, Gillman, Loustalot & Lutz, 1958) whereas only one was

found in the present study. A theca cell tumour of the ovary was also found in one female fed on the diet containing 2·0% YN. Such tumours have been described in untreated rats (Gilbert *et al.* 1958) and have occurred randomly in other studies in this laboratory (Gaunt, Brantom, Grasso, Creasey & Gangolli, 1972a; Gaunt, Carpanini, Grasso, Kiss & Gangolli, 1972b). Similarly uterine fibromyomas have been found in untreated rats (Franks, 1967).

It is noteworthy that these tumours did not occur at the highest level of YN treatment and have all been shown to occur spontaneously, suggesting that their presence is a random finding and cannot be attributed to the feeding of YN or soya lecithin.

A few tumours occurred in animals fed on the diet containing 6% YN. In all these cases the incidence of the finding was low. A single pancreatic adenocarcinoma was found, but such tumours have frequently been reported in untreated rats (Rowlatt, 967). Squamous carcinomas of the skin and tumours of the salivary gland were found in the treated animals, but similar tumours have been found in other studies in these laboratories (Gaunt, Carpanini, Grasso & Lansdown, 1972c) and have been described in untreated animals (Snell, 1965). Similarly the presence of lymphosarcomas, although limited to treated animals, does not suggest a carcinogenic effect on the part of YN, as these tumours have been encountered in other similar studies (Gaunt *et al.* 1972a). The presence of a single adenoma of the intestine is in keeping with the observation of Snell (1965), that tumours of the alimentary tract do occur infrequently.

Thus, although tumours were found in this study, in no case could they be taken as an indication of a carcinogenic effect of YN. On the basis of the present study, YN is not carcinogenic when fed to rats for 2 yr at dietary levels up to 6%. Similarly, no toxic effects that could be attributed to the ingestion of YN were found in this study and a no-untoward-effect level of 6% in the diet was established. This is equivalent to a daily intake of 2·5–3·0 g/kg and, applying the usual 100-fold safety factor, this would suggest an acceptable daily intake in man of 1·5–1·8 g/day for a 60-kg adult.

REFERENCES

Bullock, F. D. & Curtis, M. R. (1930). Spontaneous tumors of the rat. *J. Cancer Res.* **4**, 1.

Feuer, G. (1967). Metabolic fate of ^{32}P-labelled Emulsifier YN in rats. *Fd Cosmet. Toxicol.* **5**, 631.

Franks, L. M. (1967). Normal and pathological anatomy and histology of the genital tract of rats and mice. In *Pathology of Laboratory Rats and Mice.* Edited by E. Cotchin and F. J. C. Roe. p. 469. Blackwell Scientific Publications, Oxford.

Gaunt, I. F., Brantom, P. G., Grasso, P., Creasey, Margaret & Gangolli, S. D. (1972a) Long-term feeding study on Chocolate Brown FB in rats. *Fd Cosmet. Toxicol.* **10**, 3.

Gaunt, I. F., Carpanini, F. M. B., Grasso, P., Kiss, Ida S. & Gangolli, S. D. (1972b) Long-term feeding study on Black PN in rats. *Fd Cosmet. Toxicol.* **10**, 17.

Gaunt, I. F., Carpanini, F. M. B., Grasso, P. & Lansdown, A. B. G. (1972c). Long-term toxicity of propylene glycol in rats. *Fd Cosmet. Toxicol.* **10**, 151.

Gaunt, I. F., Grasso, P. & Gangolli, S. D. (1967). Short-term toxicity study of Emulsifier YN in rats. *Fd Cosmet. Toxicol.* **5**, 623.

Gilbert, Christine, Gillman, J., Loustalot, P. & Lutz, W. (1958). The modifying influence of diet and the physical environment on spontaneous tumour frequency in rats. *Br. J. Cancer* **12**, 56[illegible].

Joint FAO/WHO Expert Committee on Food Additives (1970). Toxicological Evaluation of Some Food Colours, Emulsifiers, Stabilizers, Anti-caking Agents and Certain Other Substances *F.A.O. Nutr. Mtg Rep. Ser.* no. 46A. WHO/Food Add./70.36.

Lehr, D. (1959). Causative relationships of parathyroid hormone to renogenic and renipr-ival cardiovascular disease. III. Gonadal, thyroid, and pancreatic hormones: Lipids and arteriosclerosis. *Ann. N.Y. Acad. Sci.* **72**, 901.

Lehr, D. (1965). Lesions of the cardiovascular system. In *The Pathology of Laboratory Animals.* Edited by W. E. Ribelin and J. R. McCoy. p. 124. Charles C. Thomas, Springfield, Ill.

Lemon, Phyllis G. (1967). Hepatic neoplasms of rats and mice. In *Pathology of Labora ory Rats and Mice.* Edited by E. Cotchin and F. J. C. Roe. p. 25. Blackwell Scientific Publications, Oxford.

Rowlatt, Ursula F. (1967). Pancreatic neoplasms of rats and mice. In *Pathology of Laboratory Rats and Mice*. Edited by E. Cotchin and F. J. C. Roe. p. 85. Blackwell Scientific Publications, Oxford.
Snell, Katharine C. (1965). Spontaneous lesions of the rat. In *The Pathology of Laboratory Animals*. Edited by W. E. Ribelin and J. R. McCoy. p. 241. Charles C. Thomas, Springfield, Ill.
Snell, Katharine C. (1967). Renal disease of the rat. In *Pathology of Laboratory Rats and Mice*. Edited by E. Cotchin and F. J. C. Roe. p. 105. Blackwell Scientific Publications, Oxford.

Etudes de l'émulsifiant YN: Effets de sa consommation prolongée et effets sur la reproduction chez le rat

Résumé—Des groupes de 48 rats mâles et de 48 rats femelles ont consommé de l'émulsifiant YN pendant 2 ans à raison de 0 (témoins), 2 ou 6% du régime, tandis qu'un groupe similaire recevait un régime à 4% de lécithine de soja. Ces traitements n'ont pas influé défavorablement sur la mortalité, le rythme de croissance, l'hématologie, les constituants de l'urine, la fonction de concentration des reins, les constituants du sérum et la fréquence de tumeurs. La thyroïde a augmenté de poids dans tous les groupes traités, mais ceci était la conséquence d'une plus grande fréquence d'hyperplasies des parathyroïdes, que l'on croit pouvoir imputer à des modifications spontanées de la fonction rénale combinées avec une ingestion considérable de phosphates. Une légère augmentation de la fréquence de fibroses du myocarde était aussi associée à ces hyperplasies des parathyroïdes. La consommation d'YN ou de lécithine n'a pas influé sur la fréquence et sur la gravité d'autres modifications histopathologiques.

Une étude portant sur deux générations et au cours de laquelle les animaux parents recevaient soit 0, soit 6% d'YN pendant les 13 semaines précédant l'accouplement n'a pas révélé d'effets sur la fécondité, le nombre de jeunes et la croissance de ceux-ci pendant la lactation. Aucune anomalie n'a été constatée.

Langzeit-Fütterungs- und Vermehrungsuntersuchungen mit Emulgator YN an Ratten

Zusammenfassung—Emulgator YN wurde 2 Jahre lang an Gruppen von 48 männlichen und 48 weiblichen Ratten in Konzentrationen von 0 (Kontrolle), 2 oder 6% im Futter verabreicht. Eine entsprechende Gruppe von Ratten erhielt Futter mit 4% Sojalecithin. Dies beeinflusste nicht nachteilig die Mortalität, die Geschwindigkeit der Körpergewichtszunahme, die Hämatologie, die Urinbestandteile, die Konzentrationsfähigkeit der Nieren, die Serumbestandteile und die Häufigkeit von Tumoren. Das Gewicht der Schilddrüse nahm bei allen Gruppen, an die Emulgator YN verfüttert wurde, zu, aber dies war das Ergebnis einer vermehrten Häufigkeit parathyreoidealer Hyperplasie, die spontanen Veränderungen in den Nieren, kombiniert mit erhöhter Phosphataufnahme, zugeschrieben wurde. Eine leicht vermehrte Häufigkeit myocardialer Fibrosis war ebenfalls mit der parathyreoidealen Hyperplasie verbunden. Die Häufigkeit und Schwere anderer histopathologischer Veränderungen wurde durch die Zugabe von YN oder Lecithin zum Futter nicht beeinflusst.

Eine sich über zwei Generationen erstreckende Untersuchung, bei der die Tiere der Elterngeneration 13 Wochen lang vor der Paarung Futter erhielten, in dem 0 oder 6% YN enthalten war, zeigte keinen Einfluss auf die Fruchtbarkeit, die Zahl der Jungen oder das Wachstum der Jungen während der Laktation, und es wurden keine Anomalien beobachtet.

Die von nachteiligen Wirkungen freie Konzentration von YN bei dieser Untersuchung war 6%, was einer Aufnahme durch die Ratte von 3 g/kg/Tag ungefähr äquivalent ist.

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Metabolism of the Phenolic Antioxidant 3,5-Di-*tert*-butyl-4-hydroxyanisole (Topanol 354). I. Excretion and Tissue Distribution in Man, Rat and Dog

J. W. DANIEL*, T. GREEN and P. J. PHILLIPS†

Imperial Chemical Industries Ltd., Industrial Hygiene Research Laboratories, Alderley Park, Cheshire, SK10 4TJ

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Abstract—The rate and route of excretion of radioactivity have been investigated after the administration of a single oral dose of the foodstuff antioxidant, 3,5-di-*tert*-butyl-4-hydroxyanisole (Topanol 354), labelled with carbon-14, to man, rat and dog. In man, more than 80% of the radioactivity was excreted in the urine in 10 days. Rats excreted about 50% (male) and 60% (female) of the label in the urine and the remainder in the faeces in 8 days. Dogs excreted 26–38% of the label in the urine and 39–55% in the faeces in 96 hr. There was an appreciable biliary excretion of radioactivity in rats (40% in 48 hr) and dogs (87% in 24 hr). The concentration of the antioxidant was also measured in the adipose tissue and liver of rats fed on diets containing 0·1 or 0·6% Topanol 354 for 28 days. In both tissues the concentration rapidly reached a fluctuating plateau and when these animals were returned to a normal diet, the half-life of the antioxidant in fat was found to be 4–6 days.

INTRODUCTION

It is generally accepted that toxicological studies on a chemical to be used as a food additive should include an investigation of its metabolic fate in those species selected for toxicological evaluation. It is desirable that, wherever possible, information on metabolic fate in man should also be available.

3,5-Di-*tert*-butyl-4-hydroxyanisole, known as Topanol 354®, has been developed as an antioxidant for human and animal foodstuffs. This communication describes the excretion of radioactivity by rats, dogs and male human volunteers given a single oral dose of Topanol 354 labelled with carbon-14, and the tissue distribution of the antioxidant following more prolonged administration to rats.

EXPERIMENTAL

Materials. Technical grade Topanol 354 was obtained from Imperial Chemical Industries Ltd. (Petrochemicals Division), Billingham, Teesside. 3,5-Di-*tert*-butyl-4-hydroxyanisole-[^{14}C] ([^{14}C]Topanol 354) was synthesized from [U-^{14}C]phenol (The Radiochemical Centre, Amersham, Bucks). The product, m.p. 104°C and specific radioactivity 470 μCi/mmol, had a radiochemical purity of >99·9%. Solutions of the antioxidant were prepared in corn oil.

*Present address: Life Science Research, Stock, Ingatestone, Essex, CM4 9PE.

†Present address: Imperial Chemical Industries Ltd., Pharmaceuticals Division, Alderley Park, Cheshire, SK10 4TJ.

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Excretion studies

In rats. Young adult male and female Wistar rats of the specific-pathogen-free Alderley Park strain (body weight 180–200 g) were intubated with a solution of [^{14}C]Topanol 354 (1 mg/rat; 2 μCi). The five animals of each sex so treated were transferred to individual metabolism cages for the separate collection of urine and faeces. Excreta were analysed for radioactivity at intervals of 24 hr for 8 days. The excretion of radioactivity was similarly measured in two male and two female rats previously fed 0·6% Topanol 354 in the diet for 28 days.

In dogs. Three beagle dogs (12 kg body weight) supplied from an inbred strain were fasted for 18 hr and given a gelatin capsule containing a solution of [^{14}C]Topanol 354 together with the required amount of unlabelled antioxidant. The animals were housed in individual metabolism cages and urine and faeces were collected every 24 hr for 4 days. The dose levels used in these experiments were 0·5 mg/kg and 2·8 μCi, 20·0 mg/kg and 6·2 μCi, and 41·0 mg/kg and 4·5 μCi.

In man. Two male volunteers were each given a gelatin capsule containing approximately 4 μCi [^{14}C]Topanol 354 and either 2 mg (Subject RW) or 42 mg (Subject NW) of the technical-grade material. Urine was collected for 10 days following treatment.

Biliary excretion studies

In rats. Male rats were intubated with a solution containing [^{14}C]Topanol 354 (2 μCi) together with 1 or 100 mg of the unlabelled material. The bile duct was cannulated and bile was collected continuously for 48 hr. Throughout this period animals were given water containing glucose (5%) and NaCl (0·9%).

In dogs. Two beagle dogs, fitted with permanent biliary cannulae, were each given a gelatin capsule containing [^{14}C]Topanol 354 (170 mg/kg; 5·7 μCi). In the first experiment, bile was collected at hourly intervals for a total of 5 hr, commencing 1 hr after dosing. In the second experiment, bile was collected for a 10-min period 1 hr after dosing and thereafter at hourly intervals for 7 hr and again at 24 hr.

Determination of radioactivity

Faeces were homogenized in acetone (2 vols) and the homogenate was centrifuged. The supernatant was retained and portions (300–400 mg) of the air-dried acetone-insoluble material were combusted in an atmosphere of oxygen. The carbon dioxide generated was absorbed in a solution of N-NaOH (10 ml) containing ethylenediamine (10% v/v) and 0·5 ml 30% Teepol L/litre. The radioactivity in these solutions, in urine and in bile was assayed as described by Daniel & Gage (1965).

Tissue retention and accumulation of Topanol 354 in liver and adipose tissue

Experimental procedure. Groups of 20 rats (150–180 g body weight) of each sex were maintained for 28 days on a diet containing either 0·1 or 0·6% Topanol 354. Samples of liver and of mesenteric and perirenal adipose tissue were obtained from two rats of each sex killed at intervals of 3–5 days. Surviving animals were returned to the basal diet and adipose tissue was collected from two rats of each sex killed at intervals during the subsequent 14 days. Food intake was recorded daily throughout the experiment, body weight was recorded weekly and the relative liver weight was determined at autopsy.

Estimation of Topanol 354 in liver and adipose tissue. Liver (5 g) and adipose tissue (2–3 g)

were homogenized for 2 min with chloroform (10 ml). The homogenate was centrifuged and the supernatant was collected and concentrated under reduced pressure to 1–2 ml. Methanol (20 ml) was added and the precipitated fat was separated by decantation. The methanolic solution was concentrated (1 ml) and carbon tetrachloride (15 ml) was added. The solution was again concentrated and the volume was adjusted to 1 ml. Topanol 354 was assayed by gas chromatography essentially as described by Schwecke & Nelson (1964). The gas chromatograph (Model 1520, Varian Aerograph, Walton-on-Thames, Surrey) was equipped with a flame ionization detector and a 5 ft × $\frac{1}{8}$ in. external diameter stainless-steel column packed with 2% SE-30 silicone gum and 1% Tween 80 on Chromosorb W, 80–100 mesh. The column temperature was 155°C, detector and inlet temperatures were both 250°C, N_2 flow-rate was 60 ml/min and the sample size was 2 μl. Under these conditions the retention time of Topanol 354 was 180 sec. As the performance of the column was adversely affected by the accumulation of neutral fat, the column packing was replaced at regular intervals. The amount of Topanol 354 present in the sample was obtained by measuring the height of the peak and comparing it with those given by a series of standard solutions containing 10–300 μg Topanol 354/ml.

RESULTS

Excretion of radioactivity

Rats given a single oral dose of [^{14}C]Topanol 354 excreted virtually all the radioactivity within 8 days (Table 1). Although a somewhat larger proportion of the dose was excreted in the urine of female rats, there was no evidence of any sex difference in the rate of excretion. Of the rats that had been previously fed 0·6% Topanol 354 in the diet for 28 days, the two males excreted 61 and 69% of the label in the urine and 35 and 29% in the faeces in 8 days, while the corresponding values for the two females were 67 and 73% in the urine and 30 and 23% in the faeces. The increase in urinary excretion by these rats was associated with an enhanced excretion during the first 24 hr. In 12 experiments in which the radiolabelled antioxidant was fed to biliary-cannulated male rats, about 23% (range 11–29%) of the dose was excreted in the bile in 0–24 hr while an additional 17% (range 8–22%) was excreted during 24–48 hr.

Dogs excreted an average of 32% of the radioactivity in the urine and 44% in the faeces in 4 days (Table 2). In experiments with biliary-cannulated dogs, 41% of the radioactivity was excreted in the bile within 5 hr of dosing. In a second experiment, which involved the intermittent collection of bile, the total excretion was estimated from the area under the curve obtained when the radioactivity/ml bile at each collection period was plotted against time, and it was calculated that 52% of the radioactivity was excreted within 7 hr and 87% in 24 hr. Although this method of calculation is not free from error, the close similarity between the results obtained from the two experiments indicates that such errors are not appreciable. In both experiments the maximal excretion in bile occurred within 2–3 hr of dosing.

In the human experiments (Table 3), 82 and 88% of the radioactivity was excreted in the urine within 10 days. Subject NW excreted 19, 48 and 61% of the dose within 2·5, 7 and 12 hr respectively.

Table 1. *Excretion of radioactivity in the urine and faeces of rats given a single oral dose of 1 mg (2 μCi) Topanol 354*

Route of excretion	Day	Percentage of dose excreted over 8 days by									
		Males					Females				
		1	2	3	4	5	1	2	3	4	5
Urine	1	13·9	19·2	8·4	19·3	21·6	15·0	15·8	18·2	18·3	14·6
	2	17·2	25·1	28·6	16·0	15·6	28·2	19·4	20·5	19·6	16·8
	3	10·0	4·4	18·7	5·5	5·6	12·2	20·4	22·5	17·0	14·9
	4	2·7	1·6	2·5	2·7	1·5	2·0	4·6	2·2	2·5	4·7
	5	0·8	0·6	0·6	0·8	0·5	0·8	0·9	1·2	0·5	0·7
	6–8	0·6	0·8	0·5	0·5	0·6	0·7	0·7	0·7	0·7	0·7
	Total	45·2	51·7	59·3	44·8	45·4	58·9	61·8	65·3	58·6	52·4
Faeces	1	3·4	2·8	11·5	7·0	17·4	12·6	12·5	0·0	4·1	0·0
	2	29·9	29·0	3·6	30·0	22·5	11·4	7·5	21·8	20·4	21·7
	3	11·7	10·9	13·3	11·8	8·3	14 8	13·2	12·4	6·0	11·5
	4	3·6	3·1	4·5	3·3	2·8	1·9	6·3	4·1	5·1	4·8
	5	1·2	1·0	3·2	0·7	0·9	1·1	1·4	0·3	1·5	0·8
	6–8	1·4	1·4	2·4	1·4	1·4	1·4	1·4	1·4	1·4	1·4
	Total	51·2	48·2	38·5	54·2	53·3	43·2	42·3	40·0	38·5	40·2
Urine + faeces	1–8	96·4	99·9	97·8	99·0	98·7	102·1	104·1	105·3	97·1	92·6

Table 2. *Excretion of radioactivity in the urine and faeces of beagle dogs after a single oral dose of [^{14}C]Topanol 354*

Dose		Route of excretion	Percentage of dose excreted on days				
mg/kg	μCi		1	2	3	4	1–4
0·5	2·8	Urine	25·1	0·7	0·6	ND	26·4
		Faeces	50·1	4·0	1·0	ND	55·1
						Total...	81·5
20·0	6·2	Urine	20·6	9·7	2·1	0·3	32·7
		Faeces	23·6	13·6	2·1	ND	39·3
						Total...	72·0
41·0	4·5	Urine	6·2	14·2	13·5	3·9	37·8
		Faeces	—	20·5	18·6	0·0	39·1
						Total...	76·9

ND = Not determined

Table 3. *Excretion of radioactivity in the urine of male volunteers given a single oral dose of [^{14}C]Topanol 354*

Day	Percentage of dose* excreted by	
	Subject RW	Subject NW
1	69·0	69·1
2	7·1	5·6
3	3·9	3·2
4	2·7	2·1
5	1·1	ND
6	1·1	ND
7	1·5	1·5
8	ND	ND
9	ND	0·6
10	1·5	ND
Total...	87·9	82·1

ND = Not determined

*RW (body weight 70 kg) received a dose of 2·0 mg Topanol 354 (4·4 μCi) and NW (body weight 66 kg) a dose of 42·0 mg (3·9 μCi).

Retention of Topanol 354 in adipose tissue and liver

Neither the growth rate nor daily food intake was affected when rats were fed a diet containing either 0·1 or 0·6% Topanol 354. The time taken for the concentration of Topanol 354 in the adipose tissues to attain a steady state was somewhat variable (Fig. 1a, b). At the lower dose level, equilibrium was achieved within 96 hr (Fig. 1a). The average antioxidant concentration at equilibrium in male and female rats was about 12 and 47 ppm, respectively. At the higher dose level (Fig. 1b), the concentration of the antioxidant in the adipose tissues of male rats was maximal after 24 hr. The concentration declined steadily

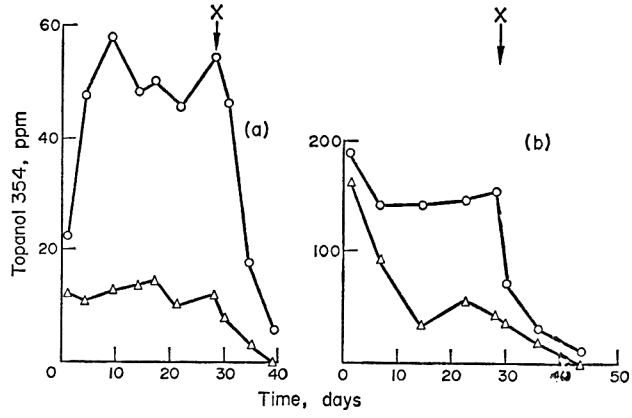

FIG. 1. Storage of Topanol 354 in the abdominal fat of male (△——△) and female (○——○) rats fed (a) 0·1% or (b) 0·6% Topanol 354 in the diet for 28 days followed (from point X) by a return to normal diet. Each point is the mean of two experiments.

during the next 14 days to a value of about 45 ppm, and this level was maintained until feeding ceased. Although a somewhat similar trend was observed in female rats, the initial decline was not so pronounced and the average concentration of antioxidant at equilibrium was about 150 ppm. When the surviving animals were returned to a basal diet, it was estimated that the concentration in the adipose tissues declined with a half-life of 4–6 days. This value was somewhat similar to that found for BHT (Daniel & Gage, 1965).

No Topanol 354 was detected in the liver of any of the male rats fed 0·1% of the antioxidant while the concentration in the female rats never exceeded 2 ppm. At the higher dose level, the antioxidant content of the liver was 2–3 ppm in male rats and 3–8 ppm in females.

Evidence was occasionally obtained for the presence of a variable amount of a second component in the extracts of adipose tissue. It was tentatively identified as 2,6-di-*tert*-butyl-*p*-benzoquinone by comparing its retention time on the gas-chromatogram (80 sec) with that of authentic material.

The effect of the continuous administration of Topanol 354 on the relative liver weight is shown in Fig. 2 (a,b). At both dietary concentrations there was an initial increase, but

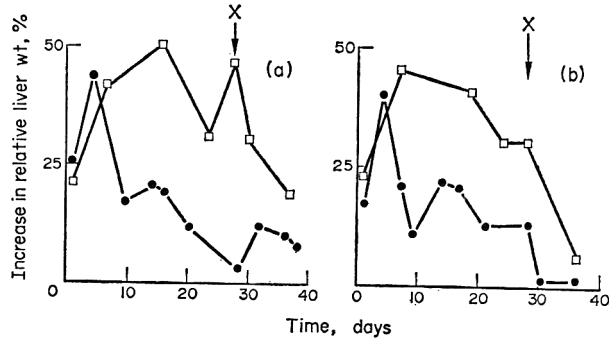

FIG. 2. Percentage increase in the relative liver weight in (a) male and (b) female rats fed 0·1 (●——●) or 0·6% (□——□) Topanol 354 for 28 days followed (from point X) by a return to normal diet. Each point is the mean of two experiments.

in contrast to those animals on the higher dose level, the relative liver weight subsequently decreased progressively in those fed 0·1% of the antioxidant.

DISCUSSION

The results of these studies indicate substantial species variation in the elimination of radioactivity after a single oral dose of [^{14}C]Topanol 354 (Tables 1–3). While the urine provides the major route of excretion in man, about one-half of the dose in rats and more than 80% in dogs is excreted in the bile. As the amount excreted in rat bile is approximately the same as that recovered from the faeces in non-cannulated rats, it would appear that little, if any, of the material excreted in rat bile is reabsorbed from the intestine. This may also be true of the dog. Although no estimate was made of the faecal excretion in man, it appears that only 10–20% of the dose is excreted by this route. If it is assumed that all of the dose is absorbed, as seems to occur in rats and dogs, then any faecal excretion in man must represent material excreted in the bile. Although the greater part of the radioactivity is excreted by man in the urine within 24 hr, a progressively diminishing excretion persists for at least 10 days. This may be due to a prolonged enterohepatic circulation of metabolites, to a slow release of the compound from the tissues, or to a combination of both. In this respect, the excretion of Topanol 354 is similar to that of the two closely-related compounds, butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) (Fig. 3). Although little is known of the factors that are involved in the biliary excretion of exogenous substances, Millburn, Smith & Williams (1967) have shown that there is a molecular-weight threshold of about 300–350 for the biliary excretion of anions in the rat and it is possible that this threshold limit is larger in man.

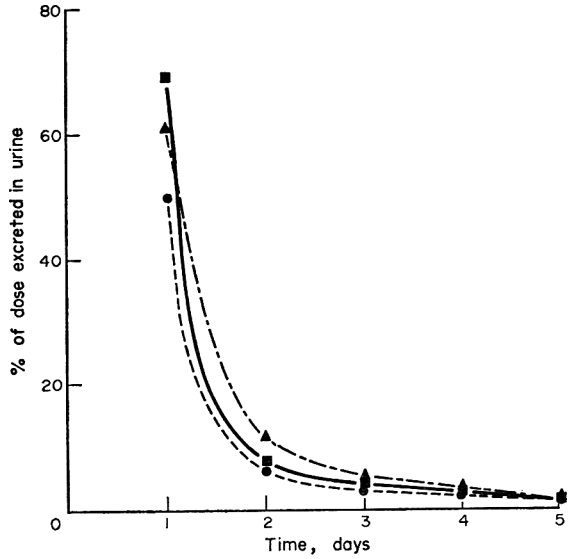

FIG. 3. Urinary excretion of Topanol 354 (■——■), BHT (●---●) and BHA (▲——▲) by human male volunteers.

Apart from differences in distribution, the results also indicate species variation in the rate of metabolism of Topanol 354. In the two human volunteers, about 70% of the dose was excreted in the urine in 24 hr, while in one of them (subject NW) 48% was excreted within 7 hr. As shown in Fig. 3, this pattern is similar to the rates of excretion of both butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT). The kinetics of the biliary excretion of radioactivity in the dog also indicate that Topanol 354 has a metabolic half-life of 7 hr or less. In contrast, less than one-half of the dose was recovered from the urine and bile of rats within 24 hr of dosing, indicating that the rate of metabolism is appreciably less in this species.

Although the continuous administration of Topanol 354 to rats at a dietary level of 0·6% caused a significant increase in the relative liver weight, the effect at the lower dose level (0·1%) was not so pronounced, since despite an initial rapid increase in both male and female rats, the weight of the liver subsequently declined steadily throughout the period of feeding and the increase was not of statistical significance by day 20. It was also observed that when [^{14}C]Topanol 354 was administered orally to male rats that had been maintained for 28 days on a diet containing 0·6% antioxidant, the proportion of the dose excreted in the urine was larger than in control rats. This was probably due to an increase in the rate of metabolism consequent upon the feeding of the antioxidant, for Topanol 354 has been shown to increase those $NADPH_2$-dependent microsomal enzymes which catalyse the oxidation of exogenous substances (Gilbert, Martin, Gangolli, Abraham & Golberg, 1969; Gillette, 1966). A similar effect observed with BHT (Daniel & Gage, 1965) was attributed by Gilbert & Golberg (1967) to increased metabolism consequent upon induction of the microsomal enzyme, BHT oxidase.

As the rate of metabolism of Topanol 354 is more rapid in man, the level of retention and accumulation is likely to be appreciably less than in the rat. This conclusion is supported by the observations of Collings & Sharratt (1970) who showed that the levels of BHT in human adipose tissue were similar to those that could be predicted from the rate of excretion of BHT by man (Daniel, Gage, Jones & Stevens, 1967).

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REFERENCES

Collings, A. J. & Sharratt, M. (1970). The BHT content of human adipose tissue. *Fd Cosmet. Toxicol.* **8,** 409.

Daniel, J. W. & Gage, J. C. (1965). The absorption and excretion of butylated hydroxytoluene (BHT) in the rat. *Fd Cosmet. Toxicol.* **3,** 405.

Daniel, J. W., Gage, J. C., Jones, D. I. & Stevens, M. A. (1967). Excretion of butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA) by man. *Fd Cosmet. Toxicol.* **5,** 475.

Gilbert, D. & Golberg, L. (1967). BHT Oxidase. A liver-microsomal enzyme induced by the treatment of rats with butylated hydroxytoluene. *Fd Cosmet. Toxicol.* **5,** 481.

Gilbert, D., Martin, A. D., Gangolli, S. D., Abraham, R. & Golberg, L. (1969). The effect of substituted phenols on liver weights and liver enzymes in the rat: Structure-activity relationships. *Fd Cosmet. Toxicol.* **7,** 603.

Gillette, J. R. (1966). Biochemistry of drug oxidation and reduction by enzymes in hepatic endoplasmic reticulum. *Adv. Pharmacol.* **4,** 219.

Millburn, P., Smith, R. L. & Williams, R. T. (1967). Biliary excretion of foreign compounds. Biphenyl, stilboestrol and phenolphthalein in the rat: Molecular weight, polarity and metabolism as factors in biliary excretion. *Biochem. J.* **105,** 1275.

Schwecke, W. M. & Nelson, J. H. (1964). Determination of antioxidants in certain food products and packaging materials by gas chromatography. *J. agric. Fd Chem.* **12,** 86.

Métabolisme de l'antioxydant phénolique 3,5-di-*tert*-butyl-4-hydroxyanisol (Topanol 354). I. Excrétion et distribution dans les tissus chez l'homme, le rat et le chien

Résumé—On a étudié chez l'homme, le rat et le chien le taux et la voie d'excrétion de la radioactivité après l'administration d'une dose orale unique d'un antioxydant employé dans l'industrie alimentaire, le 3,5-di-*tert*-butyl-4-hydroxyanisol (Topanol 354) marqué au ^{14}C. Plus de 80% de la radioactivité ont été excrétés avec l'urine et dans les 10 jours chez l'homme. En 8 jours, les rats mâles ont excrété environ 50% et les femelles environ 60% de la dose marquée avec l'urine et le reste avec les fèces. Les chiens ont excrété en 96 h de 26 à 38% de la dose marquée avec l'urine et de 39 à 55% avec les fèces. L'excrétion biliaire de la radioactivité était importante chez les rats (40% en 48 h) et chez les chiens (87% en 24 h).

On a aussi mesuré la concentration de l'antioxydant dans le tissu adipeux et dans le foie de rats qui avaient été soumis pendant 28 jours à des régimes comportant 0,1 ou 0,6% de Topanol 354. Dans ces deux tissus, la concentration atteignait rapidement un plateau variable; après que l'on eût remis les animaux au régime normal, on a pu constater que le temps de demi-vie de l'antioxydant dans la graisse était de 4 à 6 jours.

Metabolismus des phenolischen Oxydationsschutzmittels 3,5-Di-*tert*-butyl-4-hydroxyanisol (Topanol 354). I. Ausscheidung und Verteilung in den Geweben von Mensch, Ratte und Hund

Zusammenfassung—Die Geschwindigkeit und der Weg der Ausscheidung von Radioaktivität wurden nach der Verabreichung einer einzelnen oralen Dosis des Lebensmittel-Oxydationsschutzmittels, 3,5-Di-*tert*-butyl-4-hydroxyanisol (Topanol 354), markiert mit ^{14}C, an Mensch, Ratte und Hund untersucht. Der Mensch schied über 80% der Radioaktivität innerhalb von 10 Tagen mit dem Urin aus. Ratten schieden etwa 50% (männliche Tiere) und 60% (weibliche Tiere) der Markierung mit dem Urin und den Rest mit den Faeces innerhalb von 8 Tagen aus. Hunde schieden innerhalb von 96 Stunden 26–38% der Markierung mit dem Urin und 39–55% mit den Faeces aus. Die Ausscheidung von Radioaktivität mit der Galle war bedeutend bei Ratten (44% in 48 Stunden) und Hunden (87% in 24 Stunden). Die Konzentration des Oxydationsschutzmittels wurde auch im Fettgewebe und in der Leber von Ratten, die 28 Tage lang Futter mit einem Gehalt von 0,1 oder 0,6% Topanol 354 erhalten hatten, bestimmt. In beiden Geweben erreichte die Konzentration rasch eine wechselnde Höhe, und wenn diese Tiere wieder normal Futter erhielten, betrug die Halbwertszeit des Oxydationsschutzmittels im Fett 4–6 Tage.

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Metabolism of the Phenolic Antioxidant 3,5-Di-*tert*-butyl-4-hydroxyanisole (Topanol 354). II. Biotransformation in Man, Rat and Dog

J. W. DANIEL*, T. GREEN and P. J. PHILLIPS†

Imperial Chemical Industries Ltd., Industrial Hygiene Research Laboratories, Alderley Park, Cheshire, SK10 4TJ

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Abstract—The biotransformation of the phenolic antioxidant 3,5-di-*tert*-butyl-4-hydroxyanisole (Topanol 354) has been studied after oral administration to a human volunteer and to rats and dogs. The major urinary and biliary metabolites have been identified as the glucuronic- and/or sulphuric-acid conjugates of 2,6-di-*tert*-butylhydroquinone, 3,3-dimethyl-5-hydroxy-7-*tert*-butylcoumaran, 2,5-hydroxy-3,3-dimethyl-7-*tert*-butylcoumaran and 2-*tert*-butyl-6-[(2-hydroxy-1,1-dimethyl)ethyl]hydroquinone. Evidence has been obtained for the existence of isomeric forms of the latter.

INTRODUCTION

Appreciable differences have been found in both the rate and route of elimination of the proposed foodstuff antioxidant, 3,5-di-*tert*-butyl-4-hydroxyanisole (Topanol 354®) after oral administration to rats, dogs and man (Daniel, Green & Phillips, 1973). Such variation could be the result of qualitative and quantitative species differences in metabolism. This communication describes the isolation and characterization of the major metabolites of Topanol 354 from rat, dog and human urine and from rat and dog bile.

EXPERIMENTAL

Materials. Technical-grade Topanol 354 was obtained from Imperial Chemical Industries Ltd. Petrochemicals Division, Billingham, Teesside. 3,5-Di-*tert*-butyl-4-hydroxyanisole-[^{14}C] ([^{14}C]Topanol 354) was synthesized from [U-^{14}C]phenol (The Radiochemical Centre, Amersham Bucks.). The product, m.p. 104°C and specific radioactivity 470 μCi/mmol, had a radiochemical purity of >99·9%. β-Glucuronidase (625 Fishman units/mg), Type H1, was purchased from Sigma Chemical Co. Ltd., London.

Animals and dosing

Rats. Male rats of the Wistar-derived Alderley Park strain (specific-pathogen-free) were dosed orally with a solution of [^{14}C]Topanol 354 (5 mg/kg; 2 μCi) in corn oil. Animals were transferred to metabolism cages and urine was collected for 72 hr. In order to establish whether any change in metabolism occurred following repeated administration of the antioxidant and to provide sufficient quantities of individual metabolites for characterization, male rats were maintained for 3 wk on a diet containing 0·6% (w/w) Topanol 354. Urine

*Present address: Life Science Research, Stock, Ingatestone, Essex CM4 9PE.

†Present address: Imperial Chemical Industries Ltd., Pharmaceuticals Division, Alderley Park, Cheshire, SK10 4TJ.

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was collected throughout this period and for a further 72 hr after each animal had received a single oral dose of [^{14}C]Topanol 354 (5 mg/kg; 2 μCi) on day 22. The 48-hr bile was combined from two cannulated male rats given a single oral dose of [^{14}C]Topanol 354 (100 mg/kg; 0·5 μCi).

Dogs. Urine was collected for 48 hr from beagle dogs each given a gelatin capsule containing [^{14}C]Topanol 354 (0·5 mg/kg; 2–3 μCi) as a suspension in corn oil. Bile was obtained over 5 hr from a dog with a permanent biliary cannula after the oral administration of [^{14}C]Topanol 354 (170 mg/kg; 5·7 μCi).

Human volunteer. Urine was collected for 12 hr from a human male volunteer given a gelatin capsule containing [^{14}C]Topanol 354 (0·64 mg/kg; 3·9 μCi) as a suspension in corn oil (2 ml).

Separation and identification of metabolites

Solvent fractionation of metabolites. Urine was adjusted to pH 2 with conc. HCl and extracted continuously with ether for 18 hr. After separation of the ether extract, the aqueous layer was retained as fraction F1. Acidic metabolites were separated by extraction of the ether with aqueous 5% $NaHCO_3$ and the residual ether, containing neutral metabolites, was termed F2. The $NaHCO_3$ extract was acidified (pH 2) and re-extracted with ether to give fraction F3. Bile was acidified (pH 2) and extracted continuously with ether, the ether extract being separated and retained.

Chromatographic separation of metabolites. Metabolites were spotted on to thin-layer plates of silica gel G (500 μm) and the chromatograms were developed in benzene–ethanol, 9:1 (v/v), in chloroform or in toluene. Radioactive areas were located by radioautography using either Ilford Industrial G X-ray film or Kodak Kodirex film, with contact periods of 1–14 days. To determine the amount of each individual metabolite, areas of support corresponding to radioactivity were removed and the radioactivity was measured by liquid scintillation counting (Daniel & Gage, 1965). For the purification of metabolites, the material was applied as a band to one or more thin-layer plates and, after development and localization, the radioactive component(s) were eluted from the support with methanol.

Separation of ether-soluble metabolites was achieved by gas–liquid chromatography (GLC) using a 5 ft × 0·25 in. ID stainless-steel column packed with 5% SE52 on chromosorb W/AW DMCS (80–100 mesh) or glass columns of 5% polyethylene glycol succinate (PEGS) on the same support. The instrument used was a Pye model 104 gas chromatograph equipped with a flame ionization detector (operating conditions: column temperature, 175°C; injection temperature, 200°C; N_2, 50 ml/min.

The R_F values and retention times of Topanol 354 and purified metabolites are shown in Table 1.

Measurement of spectra. Ultraviolet spectra of solutions of metabolites in methanol were measured with a Unicam SP800 recording spectrometer; infra-red spectra were determined either in chloroform solution or as potassium bromide discs, using a Perkin–Elmer 257 spectrometer. Mass spectra were obtained with an MS9 double-focusing spectrometer (Associated Electrical Industries Ltd., Manchester). The nuclear magnetic resonance spectra were obtained with a Varian HA-100 spectrometer using solutions of metabolites in either $CDCl_3$ or DMSO-d_6 and subsequently after the addition of D_2O.

Measurement of radioactivity. The radioactivity of solutions of metabolites was determined using a Packard Model 3002 liquid scintillation spectrometer as described by Daniel & Gage (1965).

Table 1. *Chromatographic characteristics of Topanol 354 and its principal metabolites*

Compound	R_F in solvent system*			Retention time (min) on column†	
	1	2	3	A	B
3,5-Di-*tert*-butyl-4-hydroxyanisole	0·80	0·79	0·58	4·5	2·0
2,6-Di-*tert*-butyl-*p*-benzoquinone	0 80	0·78	0·41	2·7	1·1
3,3-Dimethyl-5-hydroxy-7-*tert*-butylcoumaran	0·66	0·49	0·17	4·5	8·0
2-*tert*-Butyl-6-[(2-hydroxy-1,1-dimethyl)ethyl]-*p*-benzoquinone: Red form	0·43	0·10	0·05	6·7	11·3
Yellow form	0·55	0·22	0·05		
2,5-Hydroxy-3,3-dimethyl-7-*tert*-butylcoumaran	0·49	0·15	0·05	8·9	—
2,6-Di-*tert*-butylhydroquinone	0·62	0·50	0·21	6·3	17·0

*Thin-layer chromatography was carried out on silica gel with the following solvent systems: 1, benzene–ethanol (9:1, v/v); 2, chloroform; 3, toluene.

†Gas–liquid chromatography was carried out at 175°C on 5 ft × 0·25 in. columns of 5% SE 52 (column A) or 5% polyethylene glycol succinate (column B), on chromosorb W/AW DMCS (80–100 mesh).

RESULTS

Excretion of radioactivity

The excretion of radioactivity, expressed as a percentage of the dose, in the urine and bile of the three species, and its distribution between fractions F1, F2 and F3 are shown in Table 2.

Table 2. *Excretion of radioactivity in urine and bile after the oral administration of [^{14}C]Topanol 354 and its distribution among fractions F1, F2 and F3*

Species	Route of excretion	Recovery of ^{14}C (% of dose)	Distribution (%) of recovered ^{14}C in fractions		
			F1	F2	F3
Rat	Urine*	57	48	6	46
	Bile	36	0	0	100
Dog	Urine	25	52	9	39
	Bile	41	0	0	100
Man	Urine	41	59	0	41

*From rats previously fed 0·6% Topanol 354 in the diet for 21 days.

Metabolites in rat urine and bile

Separation of urinary metabolites

Fraction F1, obtained from the urine of habituated rats, was refluxed with 2 N-NaOH and the solution was cooled, neutralized and extracted with ether. The aqueous solution, which contained less than 1% of the label, was discarded. The ether-soluble material was chromatographed in benzene–ethanol and three radioactive components were detected with R_F values of 0·29, 0·43, 0·55.

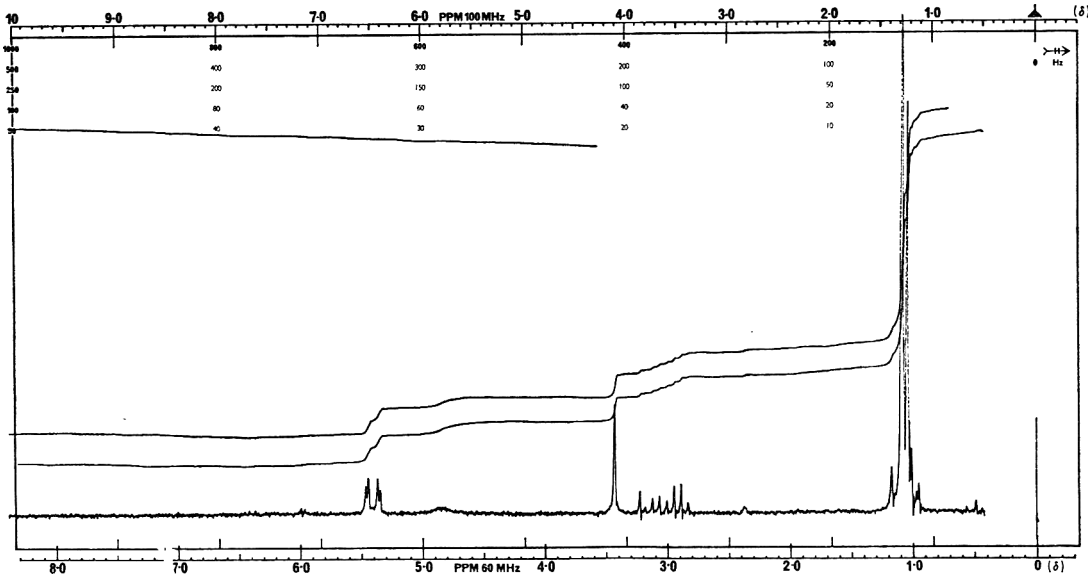

FIG. 1a. Nuclear magnetic resonance spectrum of metabolite III.

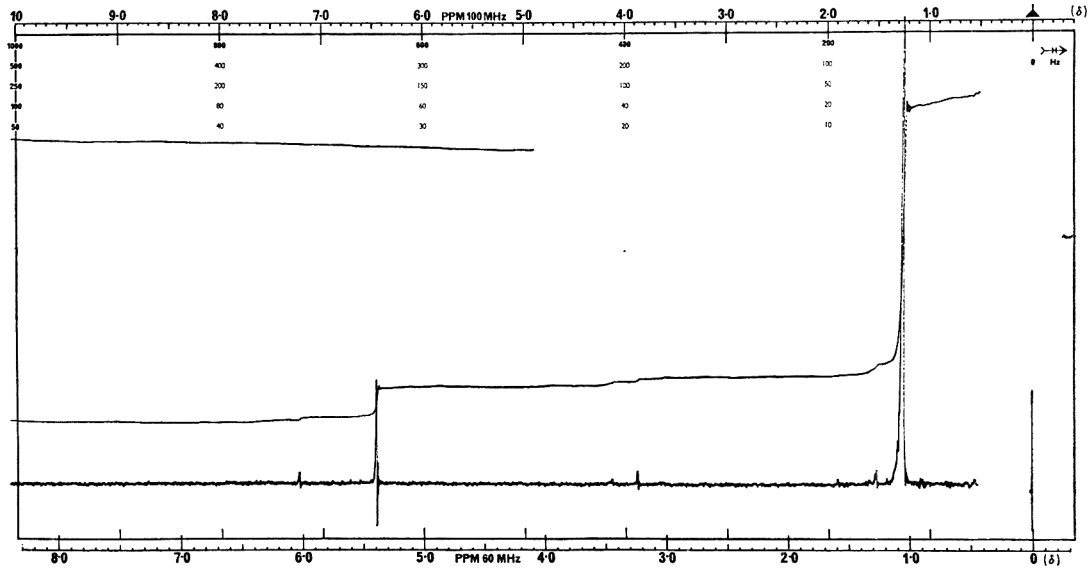

FIG. 1b. Nuclear magnetic resonance spectrum of metabolite I.

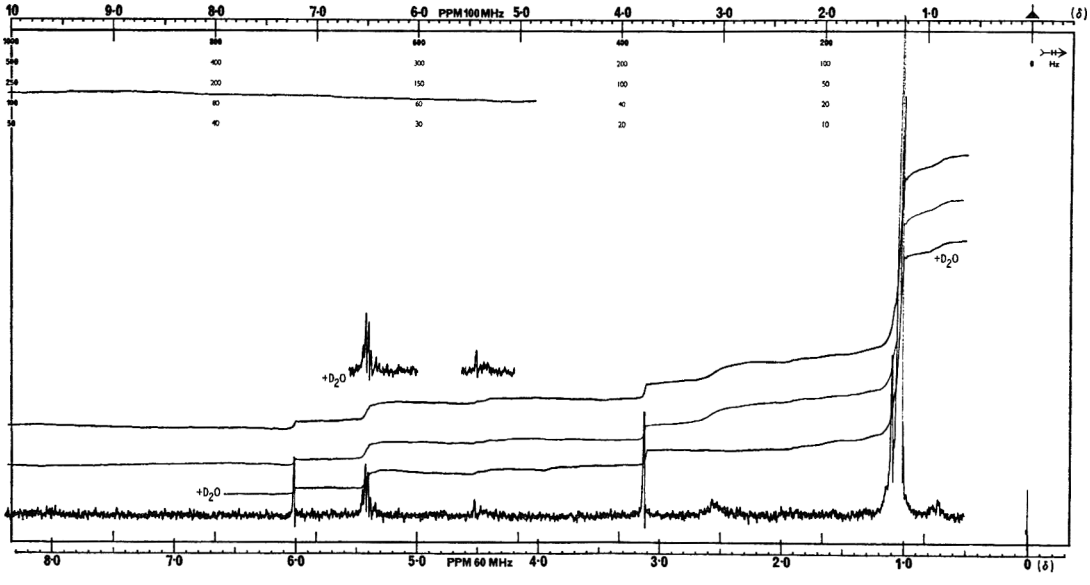

FIG. 1c. Nuclear magnetic resonance spectrum of metabolite II.

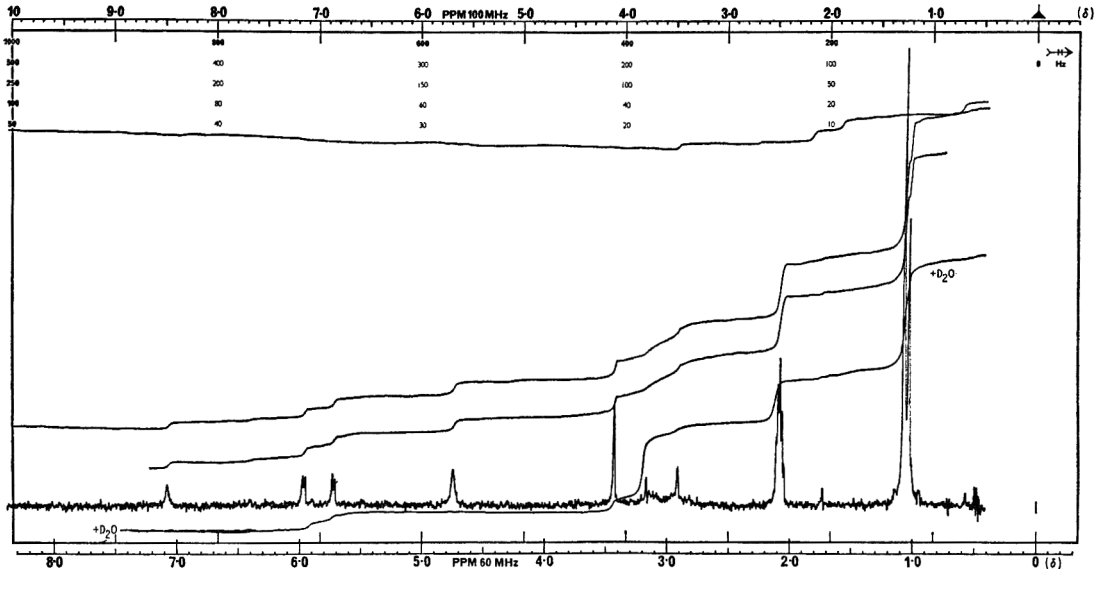

FIG. 1d. Nuclear magnetic resonance spectrum of metabolite V.

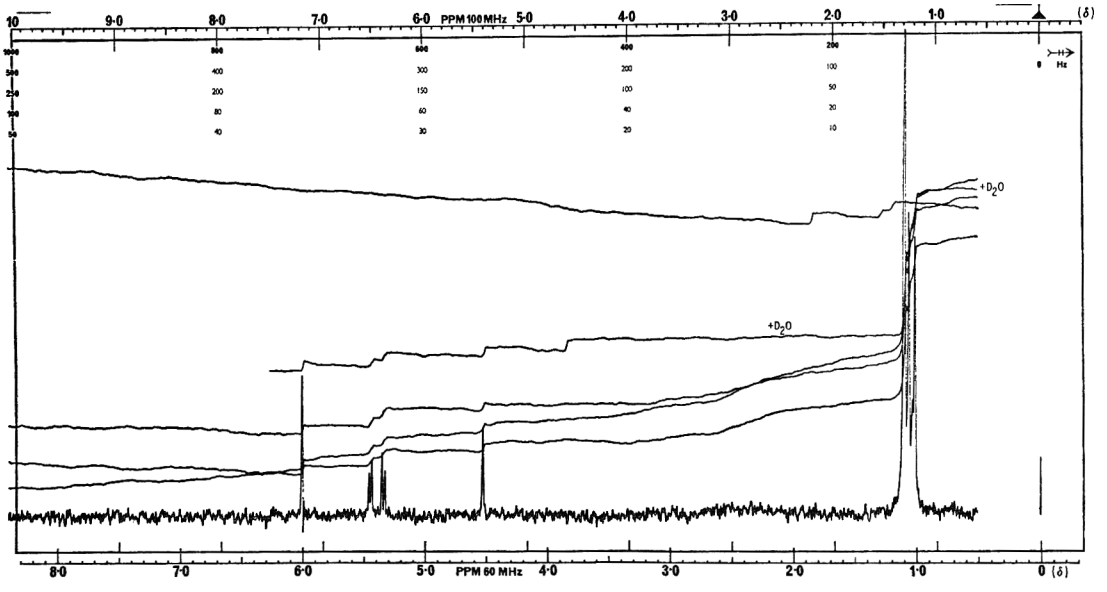

FIG. 1e. Nuclear magnetic resonance spectrum of metabolite IV.

F2 was chromatographed in the same solvent. The distribution of metabolites was similar to that in F1, except for the absence of the slower-moving component, R_F 0·29.

F3 gave a positive reaction for glucuronic acid when heated with a solution of naphthoresorcinol (0·2%, w/v) and trichloroacetic acid (33%, w/v). The extract was concentrated, and the residue was dissolved in 0·2 M-acetate buffer (20 ml) and incubated at 37°C with β-glucuronidase (100 units/ml) until all the radioactivity was recovered as ether-soluble neutral material. Chromatography of the extract in benzene–ethanol revealed the presence of five major metabolites with R_F values of 0·43, 0·49, 0·55, 0·66 and 0·80. GLC on PEGS indicated the presence of four components with retention times of 1·1, 8·0, 11·3 and 17 min, respectively. As the distribution of metabolites in all three fractions was qualitatively similar, they were combined and the metabolites were separated by preparative thin-layer chromatography in benzene–ethanol to give three radioactive bands at R_F 0·2–0·3, 0·4–0·6 and 0·6–0·8.

Identification of urinary metabolites

Band 0·6–0·8. This was found to contain three components, R_F 0·62, 0·66 and 0·80. The band at 0·66 was purified by chromatography in chloroform. The mass spectrum gave a molecular ion at m/e 220·1464, equivalent to $C_{14}H_{20}O_2$. The nuclear magnetic spectrum (Fig. 1a) was interpreted as that of 3,3-dimethyl-5-hydroxy-7-*tert*-butylcoumaran (compound III; Fig. 2). [Singlet at 1·22 ppm (6H; $-C(CH_3)_2-X$); singlet at 1·28 ppm (9H; $-C(CH_3)_3$); singlet at 4·10 ppm (2H; $-CH_2-O-Ar$); singlet at 5·82 ppm, exchangeable with D_2O (1H; —OH); multiplet at 6·50 ppm (2H; aromatic protons).] The material R_F 0·62 decomposed during purification to give a derivative that was indistinguishable from the faster moving component, R_F 0·80. The nuclear magnetic resonance spectrum of this metabolite (Fig. 1b) showed it to be 2,6-di-*tert*-butyl-*p*-benzoquinone (compound I; Fig. 2).

[Singlet at 1·25 ppm (18H; two-$C(CH_3)_3$); singlet at 6·48 ppm (2H; aromatic protons).] This structure was confirmed by comparing the infra-red spectrum with that of authentic material. The evidence suggests that this compound is an artefact obtained by oxidation of the material at R_F 0·62, which was shown by GLC to have the same retention time as 2,6-di-*tert*-butylhydroquinone.

Band 0·4–0·6. Chromatography in benzene–ethanol indicated the presence of three metabolites, R_F 0·55 (yellow), 0·43 (red) and 0·49. The mass spectrum of the faster moving component gave a molecular ion at m/e 236·1401, equivalent to $C_{14}H_{20}O_3$. The spectrum indicated the loss of ions of m/e 30, attributed to $—CH_2O$, and m/e 31, $—CH_2OH$, from the parent ion. The ultraviolet spectrum showed a single maximum at 254 nm indicative of a quinonoid configuration. The nuclear magnetic resonance spectrum (Fig. 1c) was interpreted as 2-*tert*-butyl-6-[(2-hydroxy-1,1-dimethyl)ethyl]-*p*-benzoquinone (compound II; Fig. 2). [Singlet at 1·20 ppm (6H; $—C(CH_3)_2—$); singlet at 1·22 ppm (9H; $—C(CH_3)_3$); broad band 3·00 ppm, exchangeable with D_2O (1H; —OH); singlet at 3·75 ppm (2H; $—CH_2O—$); multiplet at 6·50 ppm (2H; aromatic protons).] The two slower components were identified by comparison with material isolated from dog bile, the characterization of which is described later.

Band 0·2–0·3. The mass spectrum of the single metabolite present gave a molecular ion, m/e 262·1678, corresponding to $C_{15}H_{22}N_2O_2$. The peak at m/e 219 was derived from the parent ion by the loss of HNCO. The infra-red spectrum showed λ max 3210, 3310 and 3410 cm^{-1} attributed to $—NHCONH_2$. The nuclear magnetic resonance spectrum (Fig. 1d) was interpreted as (7-*tert*-butyl-2,3-dihydro-3,3-dimethyl-5-coumaryl)urea (compound V; Fig. 2). [Singlet at 1·20 ppm (6H; $—C(CH_3)_2$); singlet at 1·23 ppm (9H; $—C(CH_3)_3$); singlet at 4·10 ppm (2H; $—CH_2OAr$); broad singlet centred at 5·70 ppm, exchangeable for two protons, $—NH_2$; doublet at 6·85 ppm (1H; aromatic proton); doublet at 7·17 ppm (1H; aromatic proton); broad singlet at 8·50 ppm, exchangeable for 1 proton, —NH.] The imino and amino protons were similar to those in *p*-tolylurea.

The metabolites in the urine of rats given a single oral dose of Topanol 354 were similar to those found after repeated administration of the antioxidant, but there were some quantitative differences (Table 3).

Biliary metabolites

All the radioactivity present in the combined 48-hr bile was recovered as ether-soluble acidic material (Table 2). Hydrolysis with β-glucuronidase gave three metabolites with R_F values in chloroform of 0·10, 0·22 and 0·50. The first was identified as compound II (Fig. 2) on the basis of its retention time on column A and by co-chromatography with II in all three solvent systems. The material, R_F 0·50, decomposed on purification to give the benzoquinone, I (Fig. 2). The identity of the third component is considered below in connexion with biliary excretion in the dog.

Metabolites in dog urine and bile

Urinary metabolites

F1 was refluxed with 0·5 N-H_2SO_4 and the solution was cooled, neutralized and extracted with ether. F3 was hydrolysed with β-glucuronidase and the hydrolysate was extracted with ether. As thin-layer chromatography and GLC showed the two extracts to be similar, they were combined. Co-chromatography of this solution with metabolites isolated from rat urine and dog bile indicated the presence of compounds I, IIa and b, III and IV.

Biliary metabolites

All the radioactivity in the 5-hr bile was recovered as ether-soluble acidic metabolites (Table 2). Hydrolysis with β-glucuronidase gave three minor and five major metabolites with R_F values, in chloroform, of 0·10, 0·15, 0·22, 0·49 and 0·78 respectively. Co-chromatography in all three solvents showed the material with R_F 0·22, 0·49 and 0·78 to be identical with compounds II, III and I, respectively.

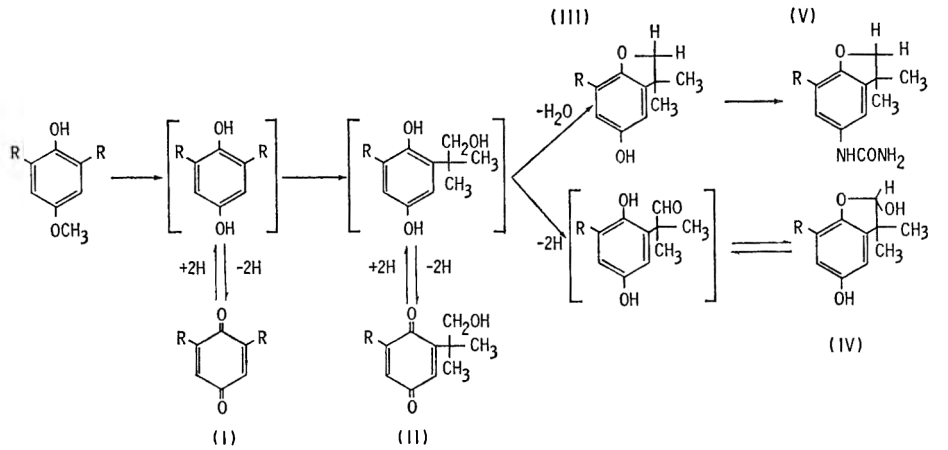

FIG. 2. The biotransformation of Topanol 354 by man, rat and dog. R = *tert*-butyl; compounds enclosed in square brackets are presumed intermediates.

The material, R_F 0·10, was red coloured and had chromatographic properties similar to those of the material present in trace amounts in both rat urine and rat bile. The GLC retention time was the same as that for metabolite II, as also were the mass and nuclear magnetic resonance spectra. The mass spectra indicated that the metabolite was not a dimeric form of compound II (Fig. 3). The only evidence that the two compounds were different was obtained from the infra-red spectra of solutions of the metabolites in chloroform. The infra-red spectra of 2,6-di-*tert*-butyl-*p*-benzoquinone in chloroform solution,

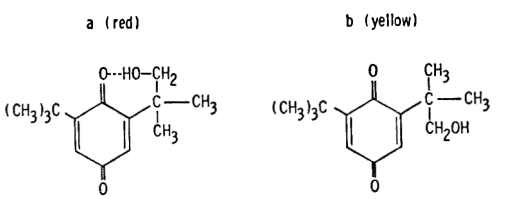

FIG. 3. Postulated structures for the isomeric forms of metabolite II.

shows only one C=O absorption attributed to asymmetric stretching at 1660 cm^{-1}. The symmetric stretch is not seen because the molecule is symmetrical and the dipole changes cancel each other. If asymmetry affecting the carbonyl group is introduced, the symmetrical stretch is seen at 1710 cm^{-1}. The yellow form (II) showed a free —OH at 3600 cm^{-1} and a very weak intra-molecular bonded —OH absorption at 3400 cm^{-1}. Asymmetrical C=O

stretching was present (ν, 1660 cm^{-1}). The red form showed strong intramolecular —OH bonding (3400 cm^{-1}) and both asymmetric and symmetric carbonyl stretching (ν, 1660 cm^{-1} and 1710 cm^{-1}). Brown (1962) has discussed the infra-red carbonyl absorption in various *p*-quinones. These findings suggest that compound II exists in two forms as shown in Fig. 3, the red form (a) showing internal H-bonding between the carbonyl at C-1 and the *ortho* substituent. This interaction may be further influenced by the hindered rotation due to the size of the *ortho* substituent.

The metabolite, R_F 0·15, was shown to have a molecular ion, m/e 236·1407, equivalent to $C_{14}H_{20}O_3$. The nuclear magnetic resonance spectrum (Fig. 1e) was interpreted as 2,5-hydroxy-3,3-dimethyl-7-*tert*-butylcoumaran (compound IV; Fig. 2). [Singlet at 1·20 ppm (3H; $—CH_3$); singlet at 1·25 ppm (3H; $—CH_3$); singlet at 1·29 ppm (9H; $—C(CH_3)_3$; broad singlet 1·80–3·50 ppm, two exchangeable protons, —OH; singlet at 5·42 ppm (1H; —H—C$<^{O—}_{O—}$ [Holder *et al.* 1970]); doublet at 6·40 ppm (1H; aromatic proton); doublet at 6·52 ppm (1H; aromatic proton).]

Table 3. *Metabolites of Topanol 354 in urine of man, rat and dog and in rat and dog bile*

	Amount excreted (% of dose) in					
	Rat			Dog		Human urine
Metabolite*	Urine†	Urine‡	Bile	Urine	Bile	
I	6·4	8·3	28·7	2·4	7·8	25·8
IIa	2·6	5·4	1·5	2·5	11·2	3·5
IIb	36·5	12·5	3·0	2·7	16·6	8·5
III	4·2	9·0	0·0	5·0	24·6	18·0
IV	6·6	6·2	0·0	5·0	14·1	5·0
V	11·2	9·2	0·0	ND	0·0	8·0

ND = Not determined

*See Figs 2 & 3 for structures.

†Urine obtained from rats maintained for 21 days on a diet containing 0·6% Topanol 354 and then given a single oral dose of [^{14}C]Topanol 354 (2 μCi).

‡Urine (72-hr) from rats given a single oral dose of [^{14}C]Topanol 354 (2 μCi).

Metabolites in human urine

F1 and F3 were treated in the same way as the fractions from dog urine, and compounds I (Fig. 2), IIa and b (Fig. 3), III and IV (Fig. 2) were identified from their chromatographic characteristics. The quantitative distribution of the primary metabolites of Topanol 354 is shown in Table 3.

DISCUSSION

The primary products of the metabolism of Topanol 354 appear to be the same in all three species studied, although there are quantitative differences (Table 3). These metabolites suggest the scheme for biotransformation shown in Fig. 2. The major metabolites in the rat are the glucuronic and sulphuric acid conjugates of 2,6-di-*tert*-butylhydroquinone and of the alcohol, compound II, produced by oxidation of one of the *tert*-butyl

substituents (Fig. 2). Although the substituted hydroquinone is also a major metabolite in man, the amount of alcohol excreted is less than in the rat, while larger amounts of the two substituted coumarans, metabolites III and IV, are produced. The dog excretes less of the hydroquinone, but is similar to the rat in the amount of alcohol formed; the excretion of the coumarans is similar to that in man. There would appear to be no significant differences in the extent of conjugation with glucuronic and sulphuric acids, assuming that F1 contains sulphate conjugates and F3 glucosiduronic acids (Table 2).

We have previously shown that while urine provides the major route of excretion of the antioxidant in man, more than 30 and 80% is excreted in the bile of rats and dogs, respectively (Daniel *et al.* 1973). In the dog, these differences are clearly related, at least in part, to the formation of the glucuronic acid conjugates of the substituted hydroquinone, the alcohol and the two substituted coumarans. In a series of publications, reviewed by Millburn (1970), Prof. R. T. Williams and his colleagues have suggested that extensive biliary excretion in rats only occurs with compounds that possess a polar anionic group and a molecular weight greater than 300–350. In the present study, analysis of rat and dog bile demonstrated the presence of only glucuronic acid conjugates, the molecular weight of each of which is above the estimated threshold value. The absence of sulphate esters from the bile may therefore be due to their lower molecular weight. As previous studies have indicated the probable absence of any significant enterohepatic circulation of metabolites of Topanol 354 (Daniel *et al.* 1973), it is concluded that little biliary excretion occurs in man. As the proportion of glucuronic acid conjugates in the urine in this species is quite large (F3; Table 2), it is possible that the molecular weight threshold for biliary excretion in man is higher than that for the rat.

This study has also demonstrated several novel transformations. Oxidation of the *tert*-butyl group to the corresponding alcohol and acid has been reported for *tert*-butylbenzene and BHT (Dacre, 1961; Daniel, Gage & Jones, 1968; Robinson & Williams, 1955). The alcohol is presumed to be the precursor of the substituted coumaran, III, while metabolite IV must be derived from the corresponding aldehyde. Although no direct evidence has been obtained for the formation of the latter, presumptive evidence for the conversion of BHT to an aldehyde has been discussed by Daniel *et al.* (1968) and by Holder, Ryan, Watson & Wiebe (1970). Spectroscopic data suggest that metabolite IV exists exclusively in the lactol form, unlike aldosterone which is known to be a mixture of the aldol and lactol forms (Shoppee, 1964). The formation of substituted coumarans has been observed following photo-irradiation of solutions of both 2,5- and 2,6-di-*tert*-butyl-*p*-benzoquinone (Orlando, Mark, Bose & Manhas, 1967; Shulgin & Kerlinger, 1965).

As previously mentioned in the Results section, the existence of isomeric forms of the alcohol, II (Fig. 3), must be due partly to the restricted rotation of the *ortho* substituent which may be further stabilized by hydrogen bonding involving the carbonyl and primary alcohol groups. The methoxy substituent was not present in any of the metabolites identified: *O*-dealkylation of aromatic ethers is a reaction which is normally catalysed by $NADPH_2$-dependent enzymes of the endoplasmic reticulum and which involves the formation of formaldehyde (Gillette, 1966). However, although the substituted quinone, I, was identified as the major product when Topanol 354 was incubated with rat-liver microsomes in the presence of $NADPH_2$, no evidence was obtained for the production of formaldehyde (J. W. Daniel, T. Green and P. J. Phillips, unpublished data 1972). This suggests that the loss of the methoxyl substituent is a consequence of a capacity of Topanol 354 to function as an antioxidant and to proceed via the formation of unstable intermediates (Hewgill &

Lee, 1968). The presence of 2,6-di-*tert*-butyl-*p*-benzoquinone in the abdominal fat of rats maintained on diets containing Topanol 354 supports this conclusion (Daniel *et al.* 1973).

In a previous paper (Daniel *et al.* 1973), we have shown that animals maintained on a diet containing the antioxidant and dosed subsequently with [^{14}C]Topanol 354 excrete more of the radioactivity in the urine than occurs with non-habituated rats. The results in Table 3 show that habituated animals excrete more of the alcohol in the urine, although the amount of the quinone excreted is unchanged. This may be due to stimulation of the enzyme responsible for oxidation of the *tert*-butyl substituent and is presumably related to the ability of Topanol 354 to stimulate $NADPH_2$-dependent microsomal enzymes (Gilbert, Martin, Gangolli, Abraham & Golberg, 1969).

Despite differences in the rate and route of excretion of the antioxidant in the three species studied, the findings indicate that the toxicology of Topanol 354 and its metabolites is likely to be similar in all these species and that close parallels may be made.

Acknowledgements—We should like to express our thanks to Dr. D. M. Ferguson for the bile-duct cannulations and to Dr. D. Greatbanks and Mr. M. J. Rix, Imperial Chemical Industries Ltd. (Pharmaceuticals Division) for the nuclear magnetic resonance and mass spectra.

REFERENCES

Brown, T. L. (1962). The infrared carbonyl absorption in some *p*-quinones and related substances. *Spectrochim. Acta* **18,** 1065.

Dacre, J. C. (1961). The metabolism of 3:5-di-*tert*.-butyl-4-hydroxytoluene and 3:5-di-*tert*.-butyl-4-hydroxybenzoic acid in the rabbit. *Biochem. J.* **78,** 758.

Daniel, J. W. & Gage, J. C. (1965). The absorption and excretion of butylated hydroxytoluene (BHT) in the rat. *Fd Cosmet. Toxicol.* **3,** 405.

Daniel, J. W., Gage, J. C. & Jones, D. I. (1968). The metabolism of 3,5-di-*tert*.-butyl-4-hydroxytoluene in the rat and in man. *Biochem. J.* **106,** 783.

Daniel, J. W., Green, T. & Phillips, P. J. (1973). Metabolism of the phenolic antioxidant 3,5-di-*tert*-butyl-4-hydroxyanisole (Topanol 354). I. Excretion and tissue distribution in man, rat and dog. *Fd Cosmet. Toxicol.* **11,** 771.

Gilbert, D., Martin, A. D., Gangolli, S. D., Abraham, R. & Golberg, L. (1969). The effect of substituted phenols on liver weights and liver enzymes in the rat: Structure-activity relationships. *Fd Cosmet. Toxicol.* **7,** 603.

Gillette, J. R. (1966). Biochemistry of drug oxidation and reduction by enzymes in hepatic endoplasmic reticulum. *Adv. Pharmacol.* **4,** 219.

Hewgill, F. R. & Lee, S. L. (1968). Oxidation of alkoxyphenols. Part XV. Autoxidation of 2- and 3-mono- and 2,5-di-t-butyl-4-methoxyphenol and related compounds. *J. chem. Soc.* (*C*) p. 1549.

Holder, G. M., Ryan, A. J., Watson, T. R. & Wiebe, L. I. (1970). The metabolism of butylated hydroxytoluene, (3,5-di-t-butyl-4-hydroxytoluene) in man. *J. Pharm. Pharmac.* **22,** 375.

Millburn, P. (1970). Factors in the biliary excretion of organic compounds. In *Metabolic Conjugation and Metabolic Hydrolysis.* Vol. 2. Edited by W. H. Fishman. p. 1. Academic Press, New York.

Orlando, C. M., Jr., Mark, H., Bose, A. K. & Manhas, M. S. (1967). Photoreactions. IV. Photolysis of *t*-butyl-substituted *p*-benzoquinones. *J. Am. chem. Soc.* **89,** 6527.

Robinson, D. & Williams, R. T. (1955). Studies in detoxication. 61. The metabolism of alkylbenzenes. *Tert*.-butylbenzene. *Biochem. J.* **59,** 159.

Shoppee, C. W. (1964). *Chemistry of the Steroids.* 2nd ed. p. 256. Butterworths, London.

Shulgin, A. T. & Kerlinger, H. O. (1965). The structures of the photochemical decomposition products of 2,5-di-*tert*-butyl-*p*-benzoquinone. *Tetrahedron Lett.* **38,** 3355.

Métabolisme de l'antioxydant phénolique 3,5-di-*tert*-butyl-4-hydroxyanisol (Topanol 354). II. Biotransformation chez l'homme, le rat et le chien

Résumé—On a étudié la biotransformation de l'antioxydant phénolique 3,5-di-*tert*-butyl-4-hydroxyanisol (Topanol 354) administré par voie orale à un sujet bénévole, à des rats et à des

chiens. Les principaux métabolites urinaires et biliaires identifiés sont les conjugués d'acide glycuronique et/ou sulfurique de la 2,6-di-*tert*-butylhydroquinone, de la 3,3-diméthyl-5-hydroxy-7-*tert*-butylcoumarine, de la 2,5-hydroxy-3,3-diméthyl-7-*tert*-butylcoumarine, et de la 2-*tert*-butyl-6-[(2-hydroxy-1,1-diméthyl)éthyl]hydroquinone. On a obtenu des preuves de l'existence de formes isomères de la dernière de ces substances.

Metabolismus des phenolischen Oxydationsschutzmittels 3,5-Di-*tert*-butyl-4-hydroxyanisol (Topanol 354). II. Biotransformation bei Mensch, Ratte und Hund

Zusammenfassung—Die Biotransformation des phenolischen Oxydationsschutzmittels 3,5-Di-*tert*-butyl-4-hydroxyanisol (Topanol 354) wurde nach der oralen Verabreichung an eine freiwillige Versuchsperson und an Ratten und Hunde untersucht. Die wesentlichen Urin- und Gallestoffwechselprodukte wurden als das Glucuron- und/oder Schwefelsäurekonjugat von 2,6-Di-*tert*-butylhydrochinon, 3,3-Dimethyl-5-hydroxy-7-*tert*-butylcumaran, 2,5-Hydroxy-3,3-dimethyl-7-*tert*-butylcumaran und 2-*tert*-Butyl-6-[(2-hydroxy-1,1-dimethyl)-äthyl]hydrochinon identifiziert. Es wurden auch Hinweise für das Existieren der Isomerformen der letzteren gefunden.

Fd Cosmet. Toxicol. Vol. 11, pp. 793–796. Pergamon Press 1973. Printed in Great Britain

Metabolism of the Phenolic Antioxidant 3,5-Di-*tert*-butyl-4-hydroxyanisole (Topanol 354). III. The Metabolism in Rats of the Major Autoxidation Product, 2,6-Di-*tert*-butyl-*p*-benzoquinone

J. W. DANIEL*, T. GREEN and P. J. PHILLIPS†

Imperial Chemical Industries Ltd., Industrial Hygiene Research Laboratories, Alderley Park, Cheshire, SK10 4TJ

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Abstract—When 2,6-di-*tert*-butyl-*p*-[^{14}C]benzoquinone (DBQ), the major autoxidation product of Topanol 354, was administered orally to rats, an average of 36% of the label was excreted in the urine and 63% in the faeces in 7 days. In studies with biliary-cannulated rats, 35% of an oral dose was recovered in the bile in 48 hr. The major urinary metabolites have been identified as glucuronic and/or sulphuric acid conjugates of 2,6-di-*tert*-butylhydroquinone (DBHQ; 11·3% of dose), 3,3-dimethyl-5-hydroxy-7-*tert*-butylcoumaran (7·2%) and 2-*tert*-butyl-6-[(2-hydroxy-1,1-di-methyl)ethyl]-*p*-benzoquinone (6·1%). The principal biliary metabolite was a glucuronic acid conjugate of DBHQ (26·7% of the dose). As the distribution and biotransformation of DBQ were similar in almost all respects to the fate of the corresponding metabolite formed from the parent antioxidant *in vivo*, it may be concluded that ingestion of the autoxidation product in the small amounts likely to be present in foods will not be associated with biological effects other than those identified after the feeding of Topanol 354.

INTRODUCTION

Frazer (1970) advocated that the safety evaluation of a food additive intended for human use should not be confined to the additive but should include those derivatives produced either by interaction of the additive with compounds in foods or by decomposition during the storage or processing of the product. The major autoxidation product of the antioxidant 3,5-di-*tert*-butyl-4-hydroxyanisole (Topanol 354®) has been identified as 2,6-di-*tert*-butyl-*p*-benzoquinone (DBQ) (R. B. Wright, personal communication 1971). The compound has also been identified as a major metabolite of the antioxidant in man, rat and dog (Daniel, Green & Phillips, 1973). This communication describes the metabolism of DBQ after oral administration to rats.

EXPERIMENTAL

Materials. 2,6-Di-*tert*-butyl-*p*-[^{14}C]benzoquinone ([^{14}C]DBQ), specific radioactivity 440 μCi/mmol was obtained from Imperial Chemical Industries Ltd. (Petrochemicals Division), Billingham, Teesside.

Animals and dosing. Adult male albino Wistar rats (specific-pathogen-free) of the Alderley Park strain were given a single oral dose of [^{14}C]DBQ (5 mg/kg; 2 μCi) as a solution in

*Present address, Life Science Research, Stock, Ingatestone, Essex, CM4 9PE.
†Present address: Imperial Chemical Industries Ltd., Pharmaceuticals Division, Alderley Park, Cheshire, SK10 4TJ.
®Trade name of Imperial Chemical Industries Ltd.

corn oil. The animals were then transferred to individual metabolism cages for the separate collection of urine and faeces. Excreta were analysed daily for radioactivity for 7 days. The bile duct was cannulated in two rats that had been dosed orally with [^{14}C]DBQ (2·5 mg/kg; 1 μCi) and bile was collected for 48 hr.

Solvent fractionation of metabolites. The combined 72-hr urines containing 31% of the radioactivity were extracted with ether and the extract was retained. The residual urine was then refluxed with 3 N-HCl for 3 hr under a N_2 atmosphere. The cooled solution was neutralized and extracted with ether. The combined 48-hr bile from two rats, containing 34% of the radioactivity, was concentrated under reduced pressure, and the residue was dissolved in 10 ml acetate buffer (pH 4·5) and incubated for 96 hr at 37°C with 2×10^4 Fishman units β-glucuronidase (Sigma Chemical Co. Ltd., London). The solution was neutralized and extracted with ether. All extracts were concentrated and reconstituted in methanol (1 ml).

Chromatography. Metabolites were chromatographed on thin-layer plates of silica gel GF (500 μm) using chloroform or benzene–ethanol (9:1, v/v). Radioactive areas were located by radioautography on X-ray film (Kodirex, Kodak Ltd., London). To determine the relative distribution of metabolites, those areas of support corresponding to radioactivity were removed and counted.

Measurement of radioactivity. The radioactivity of urine, faeces, bile and solutions of metabolites was measured as described by Daniel & Gage (1965).

RESULTS

Rats given a single oral dose of [^{14}C]DBQ excreted an average of 36% of the radioactivity in the urine and 63% in the faeces in 7 days (Table 1). In two biliary-cannulated rats, 32 and 36% of the radioactivity was excreted in the bile in 48 hr. About 95% of the radioactivity in urine and 93% of that in the bile was recovered as ether-soluble neutral metabolites after acid or enzyme hydrolysis. No ether-soluble metabolites were extracted from the urine prior to hydrolysis.

Table 1. *The excretion of radioactivity in the urine and faeces of male rats given a single oral dose of [^{14}C]DBQ*

	Percentage of dose* excreted by rat no.					
	1		2		3	
Day	Urine	Faeces	Urine	Faeces	Urine	Faeces
1	17·8	—†	6·1	25·1	12·8	—†
2	16·5	31·3	6·8	29·1	13·7	17·0
3	7·6	20·5	6·4	10·9	6·0	21·5
4	2·1	4·4	2·6	3·9	4·5	9·5
5–7	0·9	2·7	1·2	3·9	2·5	9·9
Total...	44·9	58·9	23·1	72·9	39·5	57·9

*[^{14}C]DBQ (2,6-di-*tert*-butyl-*p*-benzoquinone) was given in a dose of 2 μCi (5 mg/kg).
†No faeces.

Thin-layer chromatography of the ether-soluble neutral metabolites from urine showed a distribution similar to that obtained from the parent antioxidant (Daniel *et al.* 1973). The three major components were separated and, by co-chromatography with authentic material, their identities were established as DBQ (11·3% of the dose), 3,3-dimethyl-5-hydroxy-7-*tert*-butylcoumaran (7·2%) and 2-*tert*-butyl-6-[(2-hydroxy-1,1-dimethyl)ethyl]-*p*-benzoquinone (6·1%).

Two components were detected on chromatograms of the ether-soluble neutral metabolites in bile. These were identified as DBQ (26·7% of the dose) and 2-*tert*-butyl-6-[(2-hydroxy-1,1-dimethyl)ethyl]-*p*-benzoquinone (5·1%).

DISCUSSION

It has previously been established that DBQ is one of the principal urinary and biliary metabolites of the antioxidant 3,5-di-*tert*-butyl-4-hydroxyanisole in man, rat and dog (Daniel *et al.* 1973). The metabolite is also present in small amounts in the abdominal fat of rats maintained on diets containing the antioxidant and it is produced when the antioxidant is incubated with rat liver microsomes in the presence of $NADPH_2$ (J. W. Daniel, unpublished observations 1972). In the present study DBQ has been shown to be excreted as one or more acid-labile conjugates in urine and what is presumed to be a glucuronic acid conjugate in bile. This demonstrates that DBQ, like other quinones (Williams, 1959), is reduced to the corresponding hydroquinone prior to conjugation.

DBQ has been identified as the predominant autoxidation product of 3,5-di-*tert*-butyl-4-hydroxyanisole. As the distribution, rate of elimination and biotransformation of DBQ administered orally were essentially similar to the fate of the corresponding metabolite produced from the antioxidant *in vivo*, it may be concluded that ingestion of the small amounts of DBQ likely to be present in foods will not be associated with any biological effects other than those identified after the feeding of the parent compound. An independent evaluation of the toxicity of DBQ is therefore unnecessary.

REFERENCES

Daniel, J. W. & Gage, J. C. (1965). The absorption and excretion of butylated hydroxytoluene (BHT) in the rat. *Fd Cosmet. Toxicol.* **3**, 405.

Daniel, J. W., Green, T. & Phillips, P. J. (1973). Metabolism of the phenolic antioxidant 3,5-di-*tert*-butyl-4-hydroxyanisole (Topanol 354). II. Biotransformation in man, rat and dog. *Fd Cosmet. Toxicol.* **11**, 781.

Frazer, A. C. (1970). The need for more biochemical information in the field of food safety evaluation. In *Metabolic Aspects of Food Safety*. Edited by F. J. C. Roe. p. 1. Blackwell Scientific Publications, Oxford.

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

Métabolisme de l'antioxydant phénolique 3,5-di-*tert*-butyl-4-hydroxyanisol (Topanol 354). III. Le métabolisme de la 2,6-di-*tert*-butyl-*p*-benzoquinone, principal produit d'autoxydation, chez le rat

Résumé—On a administré de la 2,6-di-*tert*-butyl-*p*-[^{14}C]benzoquinone (DBQ), principal produit de l'auto-oxydation du Topanol 354, par voie orale à des rats et constaté qu'en moyenne 36% de la dose marquée sont excrétés avec l'urine et 63% avec les fèces dans les 7 jours. 35% d'une dose orale ont été récupérés de la bile dans les 48 h chez des rats auxquels on avait posé une canule biliaire. Les principaux métabolites urinaires identifiés sont des conjugués d'acide glycuronique et/ou sulfurique de la 2,6-di-*tert*-butylhydroquinone (DBHQ;

11,3% de la dose), la 3,3-diméthyl-5-hydroxy-7-*tert*-butylcoumarine (7,2%) et la 2-*tert*-butyl-6-[(2-hydroxy-1,1-diméthyl)éthyl)-*p*-benzoquinone (6,1%). Le principal métabolite biliaire est un conjugué d'acide glycuronique de la DBHQ (26,7% de la dose). Comme la distribution et la biotransformation de la DBQ sont semblables sous presque tous les rapports au destin du métabolite correspondant formé *in vivo* par l'antioxydant originel, on peut conclure que l'ingestion du produit d'auto-oxydation en petites quantités, telles qu'on pourrait en trouver dans les aliments, n'aurait pas d'effets biologiques autres que ceux observés après l'ingestion de Topanol 354.

Metabolismus des phenolischen Oxydationsschutzmittels 3,5-Di-*tert*-butyl-4-hydroxyanisol (Topanol 354). III. Der Metabolismus des wesentlichen Autoxydationsprodukts, 2,6-Di-*tert*-butyl-*p*-benzochinon, in Ratten

Zusammenfassung—Wenn 2,6-Di-*tert*-butyl-*p*-[^{14}C]benzochinon (DBC), das wesentliche Autoxydationsprodukt von Topanol 354, oral an Ratten verabreicht wurde, erschienen innerhalb von 7 Tagen 36% der markierten Verbindung im ausgeschiedenen Urin und 63% in den Faeces. Bei Untersuchungen von Ratten mit Gallenkanüle wurden 35% einer oralen Dosis innerhalb von 48 Stunden im Gallensaft gefunden. Die hauptsächlichen Urinstoffwechselprodukte wurden als Glucuron- und/oder Schwefelsäurekonjugate von 2,6-di-*tert*-butylhydrochinon (DBHC; 11,3% der Dosis), 3,3-Dimethyl-5-hydroxy-7-*tert*-butylcumaran (7,2%) und 2-*tert*-Butyl-6-[(2-hydroxy-1,1-dimethyl)äthyl]-*p*-benzochinon (6,1%) identifiziert. Das hauptsächliche Gallenstoffwechsel-produkt war ein Glucuronsäurekonjugat von DBHC (26,7% der Dosis). Da die Verteilung und die Biotransformation von DBC in fast jeder Hinsicht dem Schicksal des entsprechenden Metaboliten, in der sich aus dem Stammoxydationsschutzmittel *in vivo* bildete, entsprach, kann daraus geschlossen werden, dass die Aufnahme des Autoxydationsprodukts in den kleinen Mengen, deren Anwesenheit in Lebensmitteln möglich wäre, nicht mit anderen biologischen Wirkungen verbunden sein wird als denen, die nach der Verfütterung von Topanol 354 festgestellt wurden.

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Lipid and Enzyme Changes in the Blood and Liver of Monkeys Given Butylated Hydroxytoluene and Butylated Hydroxyanisole

A. L. BRANEN, T. RICHARDSON, M. C. GOEL and J. R. ALLEN

Departments of Food Science and Pathology, and Regional Primate Research Center, University of Wisconsin-Madison, Madison, Wisconsin, USA

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Abstract—When butylated hydroxytoluene (BHT) or butylated hydroxyanisole (BHA) was given to young monkeys in daily doses of 50 or 500 mg/kg/body weight for 28 days, few significant alterations occurred in the lipid and enzyme levels of the plasma and liver. In fact, many changes induced by corn oil were prevented by BHA. Total cholesterol levels in plasma and liver were significantly lowered by treatment with either BHA or BHT in doses of 500 mg/kg/day, although BHA was more effective. Treatment with either BHA or BHT at 50 mg/kg/day significantly lowered liver cholesterol, but only BHT significantly lowered plasma cholesterol at this dosage level. Lipid-phosphorus levels in the plasma were significantly increased by 500 mg BHA or BHT/kg/day and all animals receiving this dose and the lower dose (50 mg/kg/day) had lowered cholesterol:lipid-phosphorus ratios in the plasma and liver. A possible relationship between large doses of BHA and BHT, the level of dietary vitamin E and the type and level of dietary lipid is suggested in respect of their role in lipid metabolism in primates.

INTRODUCTION

Butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA) are used extensively as food antioxidants and are thus consumed daily by man. Numerous toxicological studies in rodents have indicated changes in the lipid patterns of blood and liver associated with the feeding of antioxidants, particularly BHT. The most marked changes occur in serum cholesterol and phospholipid levels. Day, Johnson, O'Halloran & Schwartz (1959) reported increased serum cholesterol in female rats fed a diet containing 0·1% BHT and increased serum cholesterol and phospholipids in those fed a diet containing 0·5% BHT. Male rats used in this study did not show any increase in serum cholesterol when fed the 0·1% BHT diet but did show an increase when the 0·5% BHT diet was fed. Similar results were reported by Frawley, Kohn, Kay & Calandra (1965) who found an elevation in serum cholesterol levels in male and female rats fed a diet containing 0·3% BHT. Rats treated with 5 g BHT/kg/day for 14 days were reported by Gaunt, Gilbert & Martin (1965) to show elevated levels of serum cholesterol and phospholipid, which returned to normal after administration ceased. The latter authors reported that liver phospholipid and cholesterol levels remained constant throughout the experiment. However, Pascal (1970) found a 33% decrease in hepatic neutral fat when rats were fed diets containing 0·1–0·5% BHT, and numerous other studies have shown that antioxidants inhibit fatty livers induced by ethanol or carbon tetrachloride (Di Luzio & Costales, 1965).

The mechanism of BHT's effects on lipid levels was studied by Johnson & Holdsworth (1968), who injected rats with acetate-1-^{14}C. BHT given alone increased the rate of synthesis

and turnover of body and liver fatty acids, but when 0·5% BHT was fed with lard the rate of synthesis of body and liver fatty acids were reduced. In both cases cholesterol levels were increased. The authors postulated that BHT caused its effects by increasing the availability of acetyl-coenzyme A. Other studies have indicated changes in liver oxidative enzymes, which could account in part for the changes in lipid levels. Sporn & Dinu (1967) found a reduced oxygen uptake, with succinate as substrate, by livers from rats fed 0·01–0·1% BHA, although higher levels of BHA (0·1–1·0%) increased oxygen uptake. Pascal & Terroine (1969) found decreased hepatic cytochrome oxidase in rats fed 0·05% BHT, and Slater & Torriella (1971) reported that propyl gallate inhibited NADPH-linked cytochrome *c* reductase.

In an attempt to clarify the effects of antioxidants on lipid levels, a series of experiments was conducted in which juvenile monkeys were intubated daily with BHA or BHT dissolved in corn oil.

EXPERIMENTAL

Animals and treatment. In conjunction with a study reported earlier (Allen & Engblom, 1972) three groups of rhesus monkeys (*Macaca mulatta*) were used. All groups consisted of sexually immature monkeys (juveniles) of both sexes, weighing approximately 2·5 kg. The experimental design is shown in Table 1. Group I consisted of two monkeys which received no corn oil or antioxidants. A liver biopsy was obtained from each of these animals and used to establish untreated control levels for liver lipids and enzymes. Of the eight monkeys in group II, two received corn oil, three received 500 mg BHA/kg/day plus corn oil and three received 500 mg BHT/kg/day plus corn oil. Group III consisted of nine monkeys, two receiving corn oil, four receiving 50 mg BHA/kg/day and three receiving 50 mg BHT/kg/day. Since the antioxidants were dissolved in corn oil at a concentration of 25% (w/w), all animals in group II received 2 ml corn oil/kg/day while those in group III received 0·2 ml. Throughout the 4-wk study, all animals were fed a nutritionally adequate diet (Purina Monkey Chow from Ralston Purina Co., St. Louis, Mo.) *ad lib.*

Table 1. *Experimental design*

Group no.	Treatment	Length of treatment (wk)	No. of animals/group	Dosage of corn oil (ml/kg/day)	Dosage of antioxidant (mg/kg/day)*
I	Untreated controls	0	2	0·0	0
II	Corn oil controls	4	2	2·0	0
	BHA	4	3	2·0	500
	BHT	4	3	2·0	500
III	Corn oil controls	4	2	0·2	0
	BHA	4	4	0·2	50
	BHT	4	3	0·2	50

*Antioxidants were dissolved in the corn oil at a concentration of 25% (w/w).

Conduct of experiment. To establish untreated control levels for blood lipids and enzymes, a 5 ml sample of blood was taken from each animal in group II prior to treatment with corn oil or antioxidants. During the treatment, 5 ml blood samples were taken weekly from all

animals in group II and similar samples were taken at the end of the 4-wk treatment period from animals in group III. After 2 wk of treatment, liver biopsies were taken from all animals in group II, following a 24-hr fast. The methods employed in the biopsy were those of Allen, Carstens & Olson (1967). After 4 wk of treatment, all animals were fasted for 24 hr and killed. Liver and blood samples were obtained at that time for biochemical analysis.

Treatment of blood samples. Immediately after the blood was drawn, a small aliquot was used for the determination of catalase activity according to the procedure of Richardson, Huddleson & Bethea (1953). The rest of the blood sample was separated into plasma and erythrocytes by the method of Turner & Rouser (1970b) and lipids were immediately extracted from the erythrocytes (Turner & Rouser, 1970a). Plasma samples were immediately frozen and stored at −20°C. Upon thawing, an aliquot of plasma was assayed for acid phosphatase (Bessey, Lowry & Brock, 1946) and lipids were extracted from the rest of the plasma (Carlson, 1963).

Preparation and assay of liver homogenates. Liver homogenates (10%) were prepared in ice-cold distilled water or isotonic buffer (0·44 M-sucrose, 0·025 M-KCl, 0·005 M-$MgCl_2$, 0·05 M-Tris HCl, pH 7·5) using a Potter–Elvehjem homogenizer. The distilled water homogenate was assayed for succinic dehydrogenase by the manometric method described by Umbreit, Burris & Stauffer (1964). An aliquot of the buffered homogenate was assayed for susceptibility to peroxidation by the method of Bieri & Anderson (1960) and lipids were extracted from the rest of the homogenate by the method of Folch, Lees & Sloane Stanley (1957).

Lipid analyses. All lipid extracts were assayed for free and total cholesterol by the ferric chloride method (Baginski & Zak, 1970), for triglycerides by the method of Carlson (1963) and for lipid phosphorus by the method of Morrison (1964).

Data evaluation. All data were subjected to statistical analysis using analysis of variance for data with a single criterion of classification and for groups with unequal replications (Steel & Torrie, 1960). Treatment means were compared using the least significant difference (Steel & Torrie, 1960).

RESULTS

The ultrastructural and gross biochemical changes in the livers of these monkeys were reported in an earlier publication by Allen & Engblom (1972). Total cholesterol levels in the plasma of monkeys given high doses of antioxidants (group II) increased initially in both control and BHT-treated monkeys, reaching a maximum after 2 wk of treatment but returning to nearly normal levels after 3 wk (Table 2). BHA-treated monkeys showed a decrease in total serum cholesterol throughout the experiment, the level reaching a minimum after 4 wk (Table 2). Although the cholesterol levels of the corn oil controls were significantly higher than untreated control levels after 2 and 4 wk, these increases were prevented by BHA treatment. In fact, cholesterol levels of animals treated with BHA were significantly lower than those treated with corn oil after 2 and 4 wk and significantly lower than untreated control levels after 4 wk. BHT was less effective in preventing these corn oil-induced increases and levels in these animals were significantly lower than the corn oil controls only after 4 wk. Since free cholesterol levels did not vary significantly during the treatment period, the alterations in total cholesterol were apparently due to changes in cholesterol ester levels. Low doses (50 mg/kg) of BHT but not of BHA lowered plasma cholesterol after 4 wk. These BHT-treated animals had significantly lower cholesterol levels than either corn oil-treated or untreated animals.

Table 2. *Plasma lipid levels of monkeys treated with BHA or BHT*

Treatment	No. of animals/ group	Stage of treatment (wk)	Total cholesterol (mg/100 ml)	Total lipid phosphorus (mg/100 ml)	Triglyceride (mg/100 ml)
Untreated controls‡	8‡	0	158·5 ± 25·9	14·67 ± 6·5	145·6 ± 47·5
Corn oil controls§	2	1	172·0 ± 39·8	15·84 ± 3·73	82·3 ± 3·1
		2	204·0 ± 31·2*	17·16	48·9 ± 6·3**
		3	134·0 ± 2·7	18·10 ± 4·27	104·6 ± 34·1
		4	198·0 ± 31·2*	21·12	82·3 ± 3·1
Corn oil controls‖	2	4	177·0 ± 0·0	8·03 ± 3·80	26·8 ± 15·2**
BHA (500 mg/kg)	3	1	138·7 ± 14·7	27·49 ± 18·81	135·0 ± 60·7
		2	148·0 ± 10·6††	43·13 ± 5·28**,††	86·0 ± 28·6
		3	130·6 ± 8·1	26·82 ± 13·42	179·5 ± 42·3†
		4	104·7 ± 23·4**,††	20·79 ± 4·20	64·5 ± 22·1*
BHA (50 mg/kg)	4	4	153·9 ± 30·3	10·04 ± 1·27	43·1 ± 13·8**
BHT (500 mg/kg)	3	1	153·0 ± 20·3	12·5 ± 3·43	83·1 ± 13·6
		2	202·0 ± 39·2*	34·54 ± 17·09**†	74·2 ± 36·0*
		3	133·0 ± 15·1	25·08 ± 12·64	96·4 ± 18·0
		4	138·0 ± 3·5††	33·42 ± 21·81	83·1 ± 20·6
BHT (50 mg/kg)	3	4	124·9 ± 13·3*†	7·96 ± 2·60	44·8 ± 26·3**

‡Untreated control values were obtained by analysis of plasma lipids of monkeys in group II 1 wk before treatment with corn oil or antioxidants.
§Corn oil controls for the 500 mg/kg group, given 2 ml corn oil/kg body weight daily.
‖Corn oil controls for the 50 mg/kg group, given 0·2 ml corn oil/kg body weight daily.
Values are given as the mean ± standard deviation except when only one animal was tested. Those marked with asterisks differ significantly from the untreated controls: $*P<0·05$; $**P < 0·01$. Those marked with daggers differ significantly from the appropriate corn oil controls: $†P < 0·05$; $††P < 0·01$.

Corn oil treatment did not alter the lipid-phosphorus levels of the plasma significantly, but high doses (500 mg/kg) of either BHA or BHT significantly increased lipid phosphorus after 2 wk of treatment (Table 2). No significant differences were evident, however, after 3 or 4 wk of treatment. Moreover, no differences were noted between corn oil-treated animals and those treated with 50 mg BHA or BHT/kg/day for 4 wk.

Monkeys receiving high doses of either BHA or BHT had a relatively low cholesterol: lipid-phosphorus ratio after 2 wk of treatment, the ratios being 10·8 for untreated controls, 3·4 for BHA-treated animals, 5·8 for BHT-treated animals and 11·9 for corn oil control animals. Both BHA- and BHT-treated animals maintained these lowered ratios throughout the remaining weeks of the study. The corn oil control animals maintained levels nearer to untreated levels throughout the feeding period, but a low ratio of 7·4 was reached after 3 wk.

After 2 wk of treatment the animals in group II, which were given high quantities of corn oil, had levels of plasma triglyceride significantly lower than the untreated control levels (Table 2). However, animals given 500 mg BHA/kg/day maintained higher plasma triglyceride levels than either corn oil control or BHT-treated animals, and, in fact, after 3 wk, BHA-treated animals had significantly higher plasma triglyceride levels than either corn oil control or BHT-treated animals. After 4 wk of treatment the levels of plasma triglyceride in groups II and III animals were significantly lower than the levels in group I

(untreated controls) but neither high nor low doses of BHA or BHT had any significant effect on these levels.

As with plasma lipids, total cholesterol levels in the erythrocytes showed a significant change after 2 wk of treatment and then returned to nearly normal levels after 3 wk (Table 3). Both BHA and BHT significantly lowered erythrocyte cholesterol below the untreated control level, but only BHT lowered the levels significantly below those of the corn oil control.

Table 3. *Total cholesterol levels in the erythrocytes of monkeys treated with 500 mg BHA or BHT/kg/day*

Treatment	No. of animals/ group	Stage of treatment (wk)	Total cholesterol (mg/100 ml)
Untreated controls‡	6	0	138·8 ± 29·1
Corn oil controls§	2	1	103·7
		2	109·1 ± 7·5
		3	131·5 ± 28·8
BHA (500 mg/kg)	3	1	67·4 ± 15·7**
		2	88·2 ± 14·3**
		3	102·8 ± 17·3*
BHT (500 mg/kg)	3	1	95·8 ± 15·0*
		2	60·7 ± 18·7**†
		3	105·7 ± 11·1*

‡Untreated control values were obtained by analysis of erythrocyte lipids of monkeys in group II 1 wk before treatment with corn oil or antioxidants.
§Given 2 ml corn oil/kg body weight daily.
Values are given as the mean ± standard deviation except when only one animal was tested. Those marked with asterisks differ significantly from untreated controls: $*P < 0{\cdot}05$; $**P < 0{\cdot}01$. That marked with a dagger differs significantly from the corn oil control: $\dagger P < 0{\cdot}05$.

Plasma acid-phosphatase and catalase levels showed no statistically significant difference between the corn oil and antioxidant-treated animals, although animals treated with 500 mg BHA/kg/day maintained a more constant level of these enzymes than either the corn oil- or BHT-treated animals. Increases in acid phosphatase after 2 wk and in catalase after 3 wk were less marked or were absent in BHA-treated animals. Treatment with 500 mg BHT/kg/day also lowered enzyme levels but not to the same degree as did BHA treatment.

As with plasma, the most statistically significant changes in liver lipids occurred in the levels of cholesterol (Table 4). Both BHA and BHT in doses of 500 mg/kg/day significantly reduced liver cholesterol after 2 wk, but only BHA maintained a significant difference from the corn oil controls after 4 wk. Both BHA and BHT given in doses of 50 mg/kg/day for 4 wk also reduced liver cholesterol, but not to the degree noted with higher doses of BHA and BHT. At 50 and 500 mg/kg/day, BHA and BHT lowered the cholesterol:lipid-phosphorus ratio of the liver below the corn oil control levels, an effect also noted in the plasma.

Liver triglyceride levels were significantly increased after 2 wk of feeding high quantities of corn oil (group II), although these levels returned to normal after 4 wk (Table 4). The

Table 4. *Composition of liver lipids of monkeys treated with BHA or BHT*

Treatment	No. of animals/ group	Total cholesterol (mg/g) at wk‡		Lipid phosphorus (mg/g) at wk‡		Triglycerides (mg/kg) at wk‡	
		2	4	2	4	2	4
Untreated controls§	2	10·47 ± 0·57		1·51 ± 0·11		15·01 ± 14·6	
Corn oil controls‖	2	9·00 ± 0·21	4·24 ± 0·59**	1·82 ± 0·12	0·83 ± 0·14*	83·4 ± 8·5**	9·3 ± 3·8
Corn oil controls¶	2	–	9·88 ± 2·51	–	2·07	–	32·7
BHA (500 mg/kg)	3	6·18 ± 0·41**†	3·15 ± 0·42**	1·59 ± 0·66	0·79 ± 0·03	43·6 ± 11·2*†	7·5 ± 5·8
BHA (50 mg/kg)	4	–	6·27 ± 1·45**†	–	1·75 ± 0·48	–	48·5 ± 6·7**
BHT (500 mg/kg)	3	6·25 ± 0·87*†	4·35 ± 0·15**	1·48 ± 0·08	1·09 ± 0·54	57·8 ± 16·0**†	19·8 ± 6·7
BHT (50 mg/kg)	3	–	5·88 ± 1·86**†	–	1·99 ± 0·40	–	36·3 ± 9·0*

‡A liver biopsy was taken from each monkey after 2 wk of daily doses of 500 mg/kg. All animals were sacrificed after 4 wk.
§Untreated controls were animals from group I (see Table 1) and received no corn oil or antioxidant.
‖Corn oil controls for the 500 mg/kg group, given 2 ml corn oil/kg body weight daily.
¶Corn oil controls for the 50 mg/kg group, given 0·2 ml corn oil/kg body weight daily.
Values are given as the mean ± standard deviation. Those marked with asterisks differ significantly from untreated controls: $*P < 0·05$; $**P < 0·01$. Those marked with a dagger differ significantly from the appropriate corn oil controls: $†P < 0·05$.

increase in triglycerides occurring after 2 wk was partially prevented by doses of 500 mg/kg/day of either BHA or BHT, and triglyceride levels in these animals were significantly lower than those in corn oil-treated animals. Treatment with small quantities of corn oil (group III) for 4 wk slightly increased liver triglycerides, although not significantly. Neither BHA nor BHT in doses of 50 mg/kg/day prevented this increase.

Liver succinic dehydrogenase was lowered by 500 mg BHA or BHT/kg/day, BHA having a more profound effect (Table 5). The levels were lower after both 2 and 4 wk but the differences were not statistically significant. *In vitro* formation of peroxides showed no difference after 2 wk between antioxidant-treated and control animals, but after 4 wk, 500 mg BHT/kg/day reduced the susceptibility of liver lipids to oxidation (Table 6).

Table 5. *Succinic dehydrogenase activity in the liver of monkeys treated with BHA or BHT*

Treatment	No. of animals/group	Q_{O_2} value at wk‡		
		0	2	4
Untreated controls§	2	22·5 ± 2·8	—	—
Corn oil controls‖	2	—	27·6 ± 1·3	25·9 ± 7·4
BHA (500 mg/kg)	3	—	16·0	18·9 ± 1·9
BHT (500 mg/kg)	3	—	20·8	20·8 ± 4·7

‡A liver biopsy was taken from each monkey after 2 wk of treatment. All animals were sacrificed after 4 wk.
§Untreated controls were animals from group I (see Table 1) and received no corn oil or antioxidant
‖Given 2 ml corn oil/kg body weight daily.
Values are given as the mean ± standard deviation, except where only one animal was tested.

Table 6. In vitro *formation of peroxides by liver homogenates of monkeys treated with BHA or BHT*

Treatment	No. of animals/group	TBA value at wk‡		
		0	2	4
Untreated controls§	2	1222·2 ± 407·0	—	—
Corn oil controls‖	2	—	725·0 ± 177·0**	942·5 ± 255·0
BHA (500 mg/kg)	3	—	721·7 ± 450·0**	971·6 ± 550·0
BHT (500 mg/kg)	3	—	636·7 ± 195·0**	433·3 ± 107·0**,††

‡A liver biopsy was taken from each monkey after 2 wk of treatment. All animals were sacrificed after 4 wk.
§Untreated controls were animals from group I (see Table 1) and received no corn oil or antioxidant.
‖Given 2 ml corn oil/kg body weight daily.
Values equal absorbance at 535 μ × 100/g liver and are given as the mean ± standard deviation. Those marked with asterisk differ significantly from untreated controls: $^{**}P < 0{\cdot}01$. Those marked with daggers differ significantly from the corn oil controls: $^{††}P < 0{\cdot}01$.

DISCUSSION

With the exception of cholesterol levels, few statistically significant alterations occurred in the lipid levels of juvenile monkeys treated with high or low doses of BHA or BHT. All treatments resulted in changes in lipid levels, but most of the changes appeared to be due

to the feeding of high quantities of corn oil. Indeed, high quantities of corn oil gave many indications of fatty-liver formation resulting in increased levels of liver triglyceride and lipid phosphorus, decreased levels of plasma triglyceride and lipid phosphorus and increased plasma acid phosphatase. All of these changes have been related to fatty-liver formation by such compounds as carbon tetrachloride (Di Luzio & Costales, 1965; Slater & Greenbaum, 1965). High doses of BHA (500 mg/kg) prevented many of these changes induced by corn oil, maintaining higher levels of plasma lipid phosphorus and triglycerides and lower levels of liver lipid phosphorus, liver triglycerides and plasma acid phosphatase. In addition, corn oil-induced increases in plasma cholesterol and catalase were prevented by BHA feeding. High doses of BHT also prevented many of these changes but never to as marked a degree as BHA. This prevention of fatty-liver formation by antioxidants has been reported by many authors and appears to be related to the prevention of lipid peroxidation and the maintenance of normal enzymatic activities (Di Luzio & Costales, 1965). The slight decrease in succinic dehydrogenase activity found in our experiments and the decrease in cytochrome oxidase reported by Pascal & Terrorne (1969) are consistent with this theory, since such decreases indicate an overall increase in hepatic antioxidant ability. The maintenance of lower catalase activity by BHA is an indication of an increase in reducing conditions in the blood stream (Paniker & Iyer, 1969). TBA values do not indicate an increased antioxidant effect on liver lipids by BHA although BHT does increase protection.

The increases in plasma lipid phosphorus reported here are in agreement with earlier findings in rodents, but the decreases in plasma cholesterol contradict earlier findings. Although BHT increased plasma cholesterol in rodents (Day *et al.* 1959; Gaunt *et al.* 1965), in our experiments with monkeys both high and low doses of BHT resulted in significantly decreased plasma and liver cholesterol. In addition, BHA, which is reported to have no effect on lipid levels in rodents (Sporn, Cucu, Dinu, Florescu, Rotoru & Sporn, 1967), resulted in significantly decreased plasma and liver cholesterol at high doses and significantly decreased liver cholesterol at low doses. The contradiction between these results and those reported earlier could be due to the differences in the ability of rodents and primates to metabolize and excrete BHA and BHT (Johnson, 1971). However, it is also possible that, again, our use of corn oil as a vehicle for the antioxidants could account for these differences. Feeding of high levels of corn oil has been reported to increase both plasma and liver cholesterol (Kritchevsky & Tepper, 1964). Such an elevation in cholesterol could result from a vitamin E deficiency, since the feeding of high levels of corn oil has been reported to result in a slight vitamin E deficiency (Horwitt, 1960) and it is known that such deficiencies are accompanied by elevations in plasma cholesterol (Alfin-Slater, 1960). It is possible that the high quantities of corn oil given to the monkeys in our experiments resulted in a slight vitamin E deficiency which subsequently elevated plasma cholesterol and led to the other lipid changes previously mentioned. Conceivably then, BHA and BHT acted to spare vitamin E and allow it to perform its normal functions in maintaining normal lipid levels. Previous work has indicated that BHT and other synthetic antioxidants can prevent some of the symptoms of a vitamin E deficiency, possibly by sparing vitamin E (Alfin-Slater, 1960). This does not rule out the possibility that the antioxidants are sparing the polyunsaturated fatty acids in the corn oil, which are also known to decrease cholesterol levels (Hutsell & Quackenbush, 1967), or that they are acting directly to alter the metabolism of lipids, as suggested by Johnson & Holdsworth (1968) and Day *et al.* (1959). In any case, it appears reasonable to conclude that high doses of BHA and BHT play some role in the lipid metabolism of primates. Further work is needed to clarify this role and to identify the

possible interrelationships between large doses of BHA and BHT, the level of dietary vitamin E and the type and level of dietary lipid.

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REFERENCES

Alfin-Slater, Roslyn B. (1960). Relation of vitamin E to lipid metabolism. *Am. J. clin. Nutr.* **8,** 445.

Allen, J. R., Carstens, Laurine A. & Olson, B. E. (1967). Veno-occlusive disease in *Macaca speciosa* monkeys. *Am. J. Path.* **50,** 653.

Allen, J. R. & Engblom, J. F. (1972). Ultrastructural and biochemical changes in the liver of monkeys given butylated hydroxytoluene and butylated hydroxyanisole. *Fd Cosmet. Toxicol.* **10,** 769.

Baginski, E. S. & Zak, B. (1970). Blood lipids. In *Gradwohl's Clinical Laboratory Methods and Diagnosis.* Edited by S. Frankel, S. Reitman and A. C. Sonnenwirth. 7th ed. p. 234. The C. V. Mosby Co., St. Louis.

Bessey, O. A., Lowry, O. H. & Brock, Mary J. (1946). A method for the rapid determination of alkaline phosphatase with five cubic millimeters of serum. *J. biol. Chem.* **164,** 321.

Bieri, J. G. & Anderson, A. A. (1960). Peroxidation of lipids in tissue homogenates as related to vitamin E. *Archs Biochem. Biophys.* **90,** 105.

Carlson, L. A. (1963). Determination of serum triglycerides. *J. Atheroscler. Res.* **3,** 334.

Day, A. J., Johnson, A. R., O'Halloran, M. W. & Schwartz, C. J. (1959). The effect of the antioxidant butylated hydroxy toluene on serum lipid and glycoprotein levels in the rat. *Aust. J. exp. Biol. med. Sci.* **37,** 295.

Di Luzio, N. R. & Costales, F. (1965). Inhibition of the ethanol and carbon tetrachloride induced fatty liver by antioxidants. *Expl mol. Path.* **4,** 141.

Folch, J., Lees, M. & Sloane Stanley, G. H. (1957) A simple method for isolation and purification of total lipides from animal tissues. *J. biol. Chem.* **226,** 497.

Frawley, J. P., Kohn, F. E., Kay, J. H. & Calandra, J. C. (1965). Progress report on multigeneration reproduction studies in rats fed butylated hydroxytoluene (BHT). *Fd Cosmet. Toxicol.* **3,** 377.

Gaunt, I. F., Gilbert, D. & Martin, D. (1965). Liver response test—V. Effect of dietary restriction on short-term feeding study with butylated hydroxytoluene (BHT). *Fd Cosmet. Toxicol.* **3,** 445.

Horwitt, M. K. (1960). Vitamin E and lipid metabolism in man. *Am. J. clin. Nutr.* **8,** 451.

Hutsell, T. C. & Quackenbush, F. W. (1967). Reduction of atherogenicity of natural fats by small additions of ethyl linoleate in the diet of the rat. *Lipids* **2,** 342.

Johnson, A. R. & Holdsworth, E. S. (1968). The effect of the antioxidant butylated hydroxy toluene on the lipid metabolism of the rat. *J. Nutr. Dietet.* **5,** 147.

Johnson, F. C. (1971). A critical review of the safety of phenolic antioxidants. *CRC Crit. Rev. Food Technol.* **2,** 267.

Kritchevsky, D. & Tepper, S. A. (1964). Influence of fatty acids on serum and liver cholesterol in rats. *Medna exp.* **10,** 345.

Morrison, W. R. (1964). A fast, simple and reliable method for the microdetermination of phosphorus in biological materials. *Analyt. Biochem.* **7,** 218.

Paniker, N. V. & Iyer, G. Y. N. (1969). Protective factors in the detoxification of hydrogen peroxide in erythrocytes. *Can. J. Biochem.* **47,** 405.

Pascal, G. (1970). Influence de l'ingestion de di-tertio-butyl-hydroxy-toluène (B.H.T.) sur la croissance corporelle et sur la composition du tissu hépatique du rat blanc. *Archs Sci. physiol.* **24,** 37.

Pascal, G. & Terroine, Thérèse (1969). Influence du butyl-hydroxy-toluène (B.H.T.) sur l'activité de la cytochrome oxydase du foie de rat. *C.r. hebd. Séanc. Acad. Sci., Paris Ser.D.* **268,** 1529.

Richardson, M., Huddleson, I. F. & Bethea, Rachel (1953). Study of catalase in erythrocytes and bacteria—I. Procedure for the determination of the catalase activity of erythrocytes. *Archs Biochem. Biophys.* **42,** 114.

Slater, T. F. & Greenbaum, A. L. (1965). Changes in lysosomal enzymes in acute experimental liver injury. *Biochem. J.* 96, 484.

Slater, T. F. & Torriella, M. V. (1971). Effects of propyl gallate and other antioxidants on rat liver microsomal nicotinamide-adenine dinucleotide phosphate-linked cytochrome *c* reductase. *Biochem. J.* **121,** 40P.

Sporn, A., Cucu, M., Dinu, Ileana, Florescu, Ileana, Rotaru, G. & Sporn, Raisa (1967). The toxicity of the food antioxidant butylated hydroxyanisole (BHA). *Igiena* **16,** 269.

Sporn, A. & Dinu, Ileana (1967). Effect of the antioxidizer butylhydroxyanisol (BHA) on the respiration and oxidative phosphorylation. *Revue Roum Biochim.* **4,** 301.

Steel, R. G. D. & Torrie, J. H. (1960). *Principles and Procedures of Statistics.* McGraw-Hill, Inc., New York.

Turner, J. D. & Rouser, G. (1970a). Precise quantitative determination of human blood lipids by thin-layer and triethylaminoethylcellulose column chromatography—I. Erythrocyte lipids. *Analyt. Biochem.* **38,** 423.

Turner, J. D. & Rouser, G. (1970b). Precise quantitative determination of human blood lipids by thin-layer and triethylaminoethylcellulose column chromatography—II. Plasma lipids. *Analyt. Biochem.* **38**, 437.
Umbreit, W. W., Burris, R. H. & Stauffer, J. F. (1964). *Manometric Techniques* 4th ed. p. 162. Burgess Publishing Co., Minneapolis.

Modifications des lipides et des enzymes dans le sang et le foie du singe après consommation d'hydroxytoluène ou d'hydroxyanisol butylés

Résumé—Les taux de lipides et d'enzymes du plasma et du foie n'ont présenté que peu d'altérations significatives chez de jeunes singes auxquels on a administré pendant 28 jours des doses quotidiennes de 50 ou de 500 mg/kg d'hydroxytoluène butylé (BHT) ou d'hydroxyanisol butylé (BHA). En fait, le BHA a prévenu de nombreuses modifications imputables à l'huile de maïs. Le traitement à raison de 500 mg/kg/jour de BHA ou de BHT a fait baisser significativement les taux de cholestérol total du plasma et du foie; le BHA était cependant plus efficace. Le traitement à 50 mg/kg/jour de BHA ou de BHT a fait baisser significativement le taux de cholestérol du foie, mais, à cette dose, seul le BHT a fait baisser significativement le taux de cholestérol du plasma. La dose de 500 mg/kg/jour de BHA ou de BHT a fait augmenter significativement les taux de phospholipides du plasma et chez tous les animaux qui recevaient cette dose et la dose moins élevée (50 mg/kg/jour), on a constaté une baisse du rapport cholestérol/phospholipides du plasma et du foie. Les auteurs suggèrent qu'il pourrait exister une relation entre les effets de fortes doses de BHA ou de BHT, le taux de vitamine E d'origine alimentaire et le taux de lipides d'origine alimentaire en ce qui concerne leur rôle dans le métabolisme des lipides chez les primates.

Lipid-und Enzymänderungen im Blut und in der Leber von Affen, die butyliertes Hydroxytoluol und butyliertes Hydroxyanisol erhielten

Zusammenfassung—Wenn butyliertes Hydroxytoluol (BHT) oder butyliertes Hydroxyanisol (BHA) 28 Tage lang in täglichen Dosen von 50 oder 500 mg/kg Körpergewicht jungen Affen verabreicht wurde, traten wenige signifikante Änderungen der Lipid- und der Enzymkonzentrationen von Plasma und Leber ein. Viele von Maisöl induzierte Änderungen wurden sogar durch BHA verhindert. Die Cholesteringesamtkonzentrationen im Plasma und in der Leber wurden durch Behandlung entweder mit BHA oder mit BHT in Dosierungen von 500 mg/kg/Tag signifikant vermindert, wobei BHA wirksamer war. Die Behandlung mit BHA oder BHT in der Dosierung 50 mg/kg/Tag verminderte signifikant das Lebercholesterin, aber nur BHT bewirkte in dieser Dosierung eine signifikante Verminderung des Plasmacholesterins. Die Phospholipidkonzentrationen im Plasma wurden durch 500 mg BHA oder BHT/kg/Tag wesentlich erhöht, und alle Tiere, welche diese Dosis oder die geringere Dosis (50 mg/kg/Tag) erhielten, wiesen niedrigere Cholesterin:Phospholipid-Relationen in Plasma und Leber auf. Eine mögliche Beziehung zwischen den Wirkungen grosser Dosen BHA oder BHT, der Konzentration von Vitamin E im Futter und der Art und Konzentration des Lipids im Futter wird in bezug auf deren Rolle im Lipidstoffwechsel der Primaten erörtert.

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The Formation of Carcinogenic Nitroso Compounds from Nitrite and Some Types of Agricultural Chemicals*

R. K. ELESPURU and W. LIJINSKY

The University of Tennessee-Oak Ridge Graduate School of Biomedical Sciences and the Carcinogenesis Program, Biology Division, Oak Ridge National Laboratory, Oak Ridge, Tennessee 37830, USA

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Abstract—Many widely used agricultural chemicals are derivatives of alkylureas and alkylcarbamic acids. All compounds of these types react with nitrite in mildly acid conditions to form a dialkylnitrosamine or an *N*-nitroso derivative, or both. The interaction of trisubstituted ureas with nitrite in mildly acid solution can lead to two products, the appropriate nitrosourea and, by nitrosative cleavage, the dialkylnitrosamine. Except with *N*-phenyldialkylurcas, the nitrosourea is the major product. Phenyldialkylureas and trisubstituted thioureas give dialkylnitrosamine as the sole *N*-nitroso product. Tetraalkylureas and bis-(dialkylthiocarbamyl)-disulphides also form dialkylnitrosamines by reaction with nitrous acid. The yields of these carcinogenic nitrosamines are significant at 37°C and at low concentrations of the tertiary amine (0·01 M). The insecticide, carbaryl, reacts with nitrous acid to form a highly mutagenic *N*-nitroso derivative.

INTRODUCTION

Many derivatives of urea and carbamic acid have found wide acceptance in agriculture and horticulture as pesticides and herbicides, because of their effectiveness and their low toxicity to higher animals. Thus dimethylphenylurea, *N*-methyl-1-naphthylcarbamate, tetramethylthiuram disulphide(bis-(dimethylthiocarbamyl)disulphide) and related compounds have been considered "safe" for widespread use, and the possibility of human exposure to trace amounts of them, either during their use or as residues in food derived from crops treated with them, has been considered of little consequence.

Information on the toxicity of these compounds has been obtained by feeding them to test animals in fairly large doses (Hartwell, 1951). Such tests do not, however, reflect one possible type of hazardous exposure of man. Since these compounds have the potential to react with nitrite in acid solution to form *N*-nitroso compounds, many of which are carcinogenic (Druckrey, Preussmann, Ivankovic & Schmähl, 1967), we undertook to investigate the reactions of some trialkylureas and *N*-alkylcarbamates with nitrous acid. These studies were not exhaustive, but were designed to determine the yields of *N*-nitroso compounds in a variety of conditions (including those to which man might be exposed), and the effect of different chemical structures (Fig. 1) on the course of the reaction.

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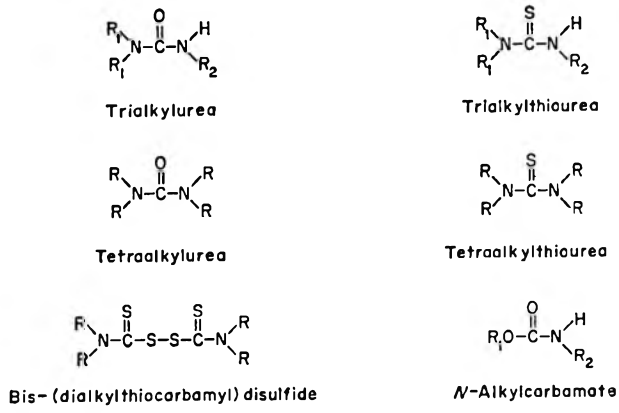

FIG. 1. Structures of relevant alkyl derivatives of urea and carbamic acid.

EXPERIMENTAL

Materials. All of the chemicals used in preparing the trialkylureas and trialkylthioureas were obtained from Aldrich Chemical Co., Milwaukee, Wisc., or from Eastman Kodak Co., Rochester, New York. The other compounds studied were all from Aldrich Chemical Co., except *N*-methylnaphthylcarbamate (carbaryl), which was from Union Carbide Corp., New York.

Preparation of ureas and thioureas

Ureas and thioureas were prepared by adding the dialkylamine dissolved in petroleum ether (30–60°C) to a solution of an equimolar amount of the appropriate alkylisocyanate or alkylisothiocyanate in petroleum ether. The amines were added under reflux and the reactions, which were sometimes violent—especially those with dimethylamine, were moderated by cooling the flasks in ice. The reactions were completed by boiling the solutions gently for 2 hr on a water bath. The trialkylureas invariably separated as crystalline solids or as oils, which crystallized on cooling. They were filtered off, washed with petroleum ether and dried to solids, which were sometimes deliquescent (dimethylethylurea and diethylmethylurea). The yields are given in Table 1, together with the melting points. The structures of all the compounds were confirmed by mass spectrometry.

Preparation of nitrosotrialkylureas

The nitroso derivatives of the trialkylureas were prepared for use as standards (and for nitrosation studies) by dissolving the trialkylureas (0·1 mol) in water containing 0·2 mol HCl, cooling in an ice bath, and slowly adding 0·2 mol sodium nitrite. There was a vigorous reaction and a light-brown oil separated. After standing for 30 min in the ice bath, the solution was extracted twice with an equal volume of methylene chloride. The methylene chloride was removed at room temperature in a stream of nitrogen and the residual oil was distilled under reduced pressure. The yield of each of the nitrosotrialkylureas was more than 83%; they boiled in a narrow range (Table 2), and mass spectral examination showed them to have the expected structure and to be virtually free of impurity.

Table 1. *Preparation and characteristics of ureas*

Compound	Starting materials	Yield (% of theoretical based on amines)	Melting point (°C)
Trialkylureas			
1,1,3-Trimethylurea	Dimethylamine + methyl isocyanate	58	66
1,1,3-Triethylurea	Diethylamine + ethyl isocyanate	80	61–62
1,1-Dimethyl-3-ethylurea	Dimethylamine + ethyl isocyanate	54	48
1,1-Diethyl-3-methylurea	Diethylamine + methyl isocyanate	79	39–40
1,1,3-Trimethylthiourea	Dimethylamine + methyl isothiocyanate	58	84–85
1,1,3-Triethylthiourea	Diethylamine + ethyl isothiocyanate	71	40–42
Dialkylphenylureas			
1,1-Dimethyl-3-phenylurea	Dimethylamine + phenyl isocyanate	57	135–137
1,1-Diethyl-3-phenylurea	Diethylamine + phenyl isocyanate	95	86–88
1,1-Dimethyl-3-phenylthiourea	Dimethylamine + phenyl isothiocyanate	55	132–133
1,1-Diethyl-3-phenylthiourea	Diethylamine + phenyl isothiocyanate	100	—

Table 2. *Preparation and characteristics of nitrosotrialkylureas*

Compound	Starting material	Yield (% of theoretical based on ureas)	Boiling point (°C)	λ_{max} (nm) (Molar absorptivity)
Nitrosotrimethylurea	Trimethylurea	90	57 (3 mm)	375 (88)–H_2O 385 (104) } CH_2Cl_2 240 (6600) }
Nitrosotriethylurea	Triethylurea	84	69–70 (3 mm)	371 (80)-H_2O 388 (109) } CH_2Cl_2 243 (7600) }
Nitrosodimethylethylurea	1,1-Dimethyl-3-ethylurea	83	67–69 (4 mm)	375 (77)-H_2O 388 (96) } CH_2Cl_2 242 (6000) }
Nitrosodiethylmethylurea	1,1-Diethyl-3-methylurea	84	68 (4 mm)	371 (80)-H_2O 384 (107) } CH_2Cl_2 240 (6100) }

It was not possible to prepare the nitroso derivatives of the trialkylthioureas in this way. Although there was a vigorous reaction when sodium nitrite was added to the acidified solution of the trialkylthiourea, no oil separated. When acetic acid was used instead of HCl, an oil did separate, but the mass spectrum was not that of a nitrosotrialkylthiourea. The UV-absorption spectrum appeared to be that of a nitrite ester (and indeed the compounds smelled of "nitrous gases"), and we speculate that the compounds were nitrite esters of the pseudo-trialkylthioureas.

Reactions of ureas and carbamates with nitrite

Reactions were carried out in an aqueous solution containing the compound to be nitrosated together with a known molar ratio of sodium nitrite (1–8 mols/mol urea or carbamate) and acetic acid sufficient to bring the pH to about 3·5 (0·5 or 1·0 ml acetic acid/20 ml of solution). In a few cases, reactions were carried out at higher pH by substituting boric acid for acetic acid. Usually reactions were allowed to proceed for specific times at 37°C in a constant-temperature bath, but in some instances the solutions were heated under reflux in a water bath at 90°C.

The trialkylurea reaction mixtures were divided into two equal parts. One half was extracted immediately with 2 × 2 vols methylene chloride, and the nitrosotrialkylurea present was estimated by ultraviolet absorption spectrometry after evaporation of the solvent in a stream of nitrogen to a convenient volume. The other half of the reaction mixture was made strongly alkaline by addition of a few pellets of sodium hydroxide (causing decomposition of the nitrosamide into a dialkylamine and diazoalkane) and was extracted with 2 × 2 vols methylene chloride for estimation of the dialkylnitrosamine. The methylene chloride extract was back-extracted with 0·1 vol 5 N-HCl to remove amines present, and was evaporated in a stream of nitrogen at room temperature to about 3 ml. This solution was diluted in a volumetric flask to 5 or 10 ml (depending on the amount of nitrosamine expected) and the nitrosamine was estimated by UV-absorption spectrometry or by gas–liquid chromatography (the analyses being carried out by the Analytical Chemistry Division, ORNL). Nitrosotriethylurea was surprisingly stable in alkali and was not decomposed completely even after standing for 24 hr in alkali.

The other reaction mixtures were made alkaline and extracted with methylene chloride, and the nitrosamine was estimated by GLC.

N-Methylnaphthylcarbamate (carbaryl) reaction mixtures, which contained much undissolved solid, were extracted with 2 × 30 ml methylene chloride, back-washed with water and evaporated to dryness. The residue was dissolved in 20 ml ethanol (most of the unchanged carbaryl remaining undissolved) and the nitrosocarbaryl present was estimated from the absorption spectrum at 402 nm (with a necessary base-line correction). The molar absorptivity of nitrosocarbaryl (yellow crystals, m.p. 66–68°C) at 402 nm was 144 in ethanol.

RESULTS AND DISCUSSION

The preparation and characteristics of a series of trialkylureas and nitrosotrialkylureas are summarized in Tables 1 and 2. In general, reaction of trialkylureas with nitrite in mildly acid solution at 37°C gives rise to the expected nitrosourea in high yield. A second product, the dialkylnitrosamine, is also formed as a small but fairly constant proportion of the products (Table 3). It appears that both nitroso compounds are formed concurrently, though at different rates, at mildly acid pH. The reaction is illustrated in Fig. 2.

The nitrosative dealkylation of trialkylureas to form dialkylnitrosamines seems to be more dependent on the ratio of nitrite to urea than does the nitrosation of the urea itself (Table 3). Nitrosotrimethylurea also undergoes nitrosative dealkylation, with formation of dimethylnitrosamine (DMN) (Table 4). The yields of DMN from nitrosotrimethylurea are similar to those from trimethylurea under the same conditions of concentration and time (Tables 3 & 4). From this it can be concluded that formation of dialkylnitrosamine from a trialkylurea does not require formation of the nitrosotrialkylurea as an intermediate.

Table 3. *Reaction of trialkylureas with different nitrite concentrations*

Trialkylurea*	Concentration of nitrite (M)	pH	Time (min)	Yield of nitrosourea (%)	Yield of dialkyl nitrosamine (%)
Trimethylurea	0·1	3·8	15	32	0·13
	0·2	3·8	15	48	0·8
	0·4	3·8	15	84	1·1
	0·8	3·8	15	89	3·7
Trimethylurea	0·1	5·7	60	0·2	—
	0·2	5·7	60	0·24	—
	0·4	5·6	60	2·8	0·013
	0·8	5·3	60	12·0	0·09
Triethylurea	0·1	3·6	60	15	1·0
	0·2	3·6	60	35	2·5
	0·4	3·6	60	62	3·7
	0·8	3·6	60	74	10·0
Dimethylethylurea	0·4	3·7	60	92	1·3
Diethylmethylurea	0·4	3·7	60	93	0·1

*In 0·1 M concentration.

The trialkylthioureas and the dialkylphenylureas do not form stable nitrosoureas under these conditions, and the dialkylnitrosamines appear to be the only *N*-nitroso compounds formed.

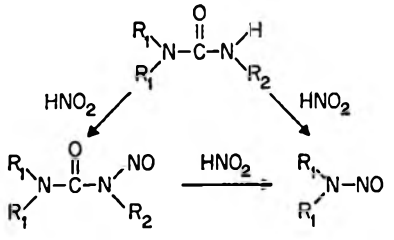

FIG. 2. Concurrent reactions of trialkylureas with nitrite in mildly acid solution.

Even under standard conditions of pH and temperature, it is obvious that several different mechanisms operate in the reaction between di-, tri- and tetraalkylureas with nitrite (Table 5). At high concentrations, 1,1-dimethylurea reacts rapidly and completely with nitrous acid to form DMN (Lijinsky, Keefer, Conrad & Van de Bogart, 1972b), probably through deamination. At lower concentrations, dimethylurea reacts more slowly and the yields of DMN are similar to those from trimethylurea and depend on the conditions used. The yield of DMN from tetramethylurea was less than 1 % under all conditions, even after heating at 90°C (Table 6).

Table 4. *Formation of dialkylnitrosamines from nitrosotrialkylureas and nitrite*

Nitrosourea*	Concentration of nitrite (M)	pH	Time (min)	Temperature (°C)	Residual 1-nitroso-1,3,3-trialkylurea (%)	Yield of dialkylnitrosamine (%)
1-Nitroso-1,3,3-trimethylurea	0	3·2	60	90		0·2
	0·4	3·2	60	90		11·0
	0·1	3·9	15	37	100	0·3
	0·2	3·9	15	37	96	0·6
	0·4	3·9	15	37	92	1·8
	0·8	3·9	15	37	87	3·4
1-Nitroso-1,3,3-triethylurea	0·4	3·8	120	37	71	3·7

*In 0·1 M concentration.

The effect of replacing the oxygen of urea compounds by sulphur on the formation of dialkylnitrosamine was examined. Tetramethylthiourea gave a much higher yield of DMN than did tetramethylurea at either high or low concentration (Table 6). However, comparison of the trialkylureas with trialkylthioureas did not show a consistent difference in the yield of dialkylnitrosamine in a given time (Table 7). The dialkylphenylureas produced somewhat higher yields of dialkylnitrosamine than the corresponding thio derivatives.

Table 5. *Reaction of nitrous acid with methylureas at 37°C*

Compound	Concentration (M)	Concentration of nitrite (M)	pH	Time (hr)	Yield of dimethyl-nitrosamine (%)
1,1-Dimethylurea	0·1	0·05	3·3	2	0
	0·1	0·1	3·3	2	0·4
	0·1	0·2	3·3	2	2·1
	0·1	0·4	3·5	2	6·7
	0·1	0·8	3·5	2	15·6
	0·1	0·4	3·7	1	3·4
	0·02	0·08	3·3	2	1·5
Trimethylurea	0·1	0·4	3·7	1	1·5
	0·02	0·08	3·3	2	1·4
Tetramethylurea	0·1	0·4	3·8	1	0·03

The fungicide tetramethylthiuram disulphide, like its tetraethyl analogue disulfiram (Lijinsky, Conrad & Van de Bogart, 1972a), reacts with nitrous acid to form the dialkylnitrosamine (Fig. 3) even at the low concentrations dictated by its slight solubility in water (Table 6). These two compounds are dialkyl carbamates and might react with nitrous acid in a way similar to dimethylphenylcarbamate, which gives small (0·14–0·27 %) but significant yields of DMN at 37°C in 1 hr.

The formation of nitrosocarbaryl from carbaryl and nitrite (Fig. 4) was studied at two concentrations and at two pH values with HCl or acetic acid as the acidifying agents. In the experiments using HCl, dimethylsulphoxide (25 %) was added to increase the solubility of the almost insoluble carbaryl. The yields of nitrosocarbaryl after 1 hr at 37°C, with occasional shaking, are given in Table 8. The percentage yields are not much reduced at a five-times lower concentration of the reactants.

Among the many unexplained findings in these experiments is the high yield of dialkylnitrosamines from dialkylphenylureas (Table 7). One possibility is that, because formation of nitrosophenyldialkylurea is not favoured (indeed, these compounds could not be identified in the reaction mixture), more nitrite was available for nitrosative dealkylation. In general, the formation of diethylnitrosamine from *N*,*N*-diethylureas was higher than that of DMN from *N*,*N*-dimethylureas (Table 7). On the other hand, in our studies of the reaction of tertiary amines with nitrous acid (Lijinsky *et al.* 1972b), there was no significant difference between ethyl and methyl compounds in the ease with which they formed nitrosamines.

The kinetics of the formation of nitrosoalkylureas from nitrite and *N*-alkylureas have

Table 6. *Reaction of tetraalkylureas and tetraalkylthiocarbamates with nitrite*

Compound	Concentration (M)	Concentration of nitrite (M)	pH	Temp. (°C)	Time (hr)	Yield of dialkylnitrosamine (%)
Tetramethylurea	0·5	2·0	3·9	90	4	0·7
	0·5	2·0	5·2	90	4	0·4
	0·1	0·4	3·6	37	1	0·03
Tetramethylthiourea	0·5	2·0	4·1	90	4	5·2
	0·5	2·0	5·3	90	4	2·1
	0·1	0·4	3·6	37	1	0·77
Tetramethylthiuram disulphide	0·01	0·04	3·3	37	3	0·1
	0·01	0·08	3·3	37	3	0·74
Disulfiram	0·01	0·04	3·3	37	3	0·08
	0·01	0·08	3·5	37	3	0·21

Table 7. *Reaction of trialkylureas and trialkylthioureas with nitrite for 1 hr*

Compound	Concentration (M)	Concentration of nitrite (M)	pH	Temperature (°C)	Yield of dialkyl-nitrosamine (%)
Trimethylurea	0·1	0·4	3·8	37	1·5
Trimethylthiourea	0·1	0·4	3·9	37	1·6
Triethylurea	0·1	0·4	3·8	37	3·7
Triethylthiourea	0·1	0·4	3·9	37	2·5
Dimethylphenylurea	0·1	0·4	4·0	90	20·6
	0·01	0·04	3·5	37	4·8
	0·01	0·04	3·3	90	27
Dimethylphenylthiourea	0·1	0·4	3·9	90	13·0
	0·01	0·04	3·5	37	0·1
	0·01	0·04	3·3	90	10
Diethylphenylurea	0·1	0·4	3·8	90	37·5
	0·1	0·4	3·8	37	2·9
	0·02	0·08	3·5	37	0·2
Diethylphenylthiourea	0·1	0·4	3·7	90	17·0
	0·1	0·4	3·7	37	0·7
	0·02	0·08	3·5	37	0·3

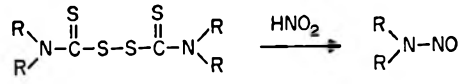

FIG. 3. Reaction of tetramethylthiuram disulphide (thiram; R = CH_3) or the tetraethyl analogue (disulfiram; R = C_2H_5) with nitrous acid to form the dialkylnitrosamine.

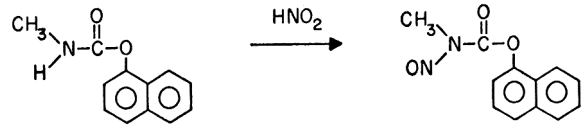

FIG. 4. Nitrosation of carbaryl by nitrous acid.

Table 8. *Formation of* N-*nitrosocarbaryl from carbaryl and nitrite in 1 hr at 37°C*

Concentration of carbaryl (M)	Concentration of sodium nitrite (M)	Volume (ml)	Acid	pH	Yield of nitrosocarbaryl	
					mg	% of theoretical
0·5	2·0	20	Hydrochloric	3·2	130	5·7
0·1	0·4	20	Hydrochloric	2·8	24	5·2
0·5	2·0	20	Acetic	3·9	245	11
0·1	0·4	20	Acetic	3·7	29	6·4

been examined (Mirvish, 1971), and it is likely that the trialkylureas behave similarly. However, the formation of dialkylnitrosamines seems to follow entirely different kinetics, and these have still to be worked out for tertiary amino compounds.

CONCLUSION

These experiments on the reaction of tertiary amino compounds with nitrous acid have indicated many facets that merit investigation. The most significant aspect, however, is that many compounds with these seemingly innocuous structures (compounds used as herbicides and pesticides that can become incidental food additives as residues on plants) react readily with nitrite at moderately acid pH to form carcinogenic nitroso compounds, among which are the well-known dialkylnitrosamines. Sander (1970) has shown the formation of carcinogenic amounts of nitrosoureas in rats fed 1,3-dimethylurea and nitrite. The nitroso derivative of *N*-methylnaphthylcarbamate (carbaryl) is a close analogue of *N*-nitrosomethylurethane, and would be expected to have similar carcinogenic and mutagenic properties.* So would the *N*-nitroso derivatives of the many other aromatic esters of *N*-methylcarbamic acid that are effective as pesticides (Epstein & Legator, 1971).

Since nitrites are present in many foods, a carcinogenic hazard to man could exist when food containing residues of certain agricultural chemicals is simultaneously present in the stomach. Most of these chemicals have been tested by long-term feeding to experimental animals but, to our knowledge, none of them has yet been fed together with nitrite. We are in the process of carrying out such tests.

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REFERENCES

Druckrey, H., Preussmann, R., Ivankovic, S. u. Schmähl, D. (1967). Organotrope carcinogene Wirkungen bei 65 verschiedenen N-Nitroso-Verbindungen an BD-Ratten. *Z. Krebsforsch.* **69**, 103.

Epstein, S. S. & Legator, M. (1971). *The Mutagenicity of Pesticides. Concepts and Evaluation.* M.I.T. Press, Cambridge, Mass.

Hartwell, J. L. (1951). Survey of Compounds Which Have Been Tested for Carcinogenic Activity. US Public Health Service Publication no 149 and Supplements, Washington, D.C.

Lijinsky, W., Conrad, Evelyn & Van de Bogart, Rosalie (1972a). Carcinogenic nitrosamines formed by drug/nitrite interactions. *Nature, Lond.* **239**, 165.

Lijinsky, W., Keefer, L., Conrad, E. & Van de Bogart, R. (1972b). Nitrosation of tertiary amines and some biologic implications. *J. natn. Cancer Inst.* **49**, 1239.

Mirvish, S. S. (1971). Kinetics of nitrosamide formation from alkylureas, *N*-alkylurethans, and alkylguanidines: Possible implications for the etiology of human gastric cancer. *J. natn. Cancer Inst.* **46**, 1183.

Sander, J. (1970). Induktion maligner Tumoren bei Ratten durch orale Gabe von N,N'-Dimethylharnstoff und Nitrit. *Arzneimittel-Forsch.* **20**, 418.

*Nitrosomethylnaphthylcarbamate is a potent mutagen in the *Haemophilus influenzae* test system, as will be described in full elsewhere.

Formation de composés nitrosés carcinogènes á partir du nitrite et de certains types de produits chimiques employés en agriculture

Résumé—Beaucoup de produits chimiques largement utilisés en agriculture sont des dérivés d'alcoylurées et d'acides alcoylcarbamiques. Tous les composés de ce type réagissent avec le nitrite en milieu légèrement acide pour former soit une dialcoylnitrosamine, soit un dérivé

N-nitroso, soit ces deux substances. L'interaction d'urées trisubstituées et du nitrite en solution légèrement acide peut donner deux produits, la nitrosurée correspondante et, par clivage nitrosatif, la dialcoylnitrosamine. Si l'on excepte les *N*-phényldialcoylurées, la nitrosurée est le produit principal. Les phényldialcoylurées et les thio-urées trisubstituées donnent de la dialcoylnitrosamine comme seul produit *N*-nitroso. Les tétra-alcoylurées et les bis-(dialcoylthiocarbamyl)-disulfures aussi forment des dialcoylnitrosamines par réaction avec l'acide nitreux. La production de ces nitrosamines carcinogènes est significative à 37°C et aux faibles concentrations de l'amine tertiaire (0,01 M). Un insecticide, le carbaryl, réagit avec l'acide nitreux pour former un dérivé *N*-nitroso fortement mutagène.

Die Bildung carcinogener Nitrosoverbindungen aus Nitrit und einigen Arten von Landwirtschaftschemikalien

Zusammenfassung—Zahlreiche vielverwendete Landwirtschaftschemikalien sind Derivate von Alkylharnstoffen und Alkylcarbaminsäuren. Alle Verbindungen dieser Arten reagieren mit Nitrit unter schwachsauren Bedingungen zu Dialkylnitrosamin oder einem *N*-Nitrosoderivat oder beidem. Die Reaktion von trisubstituierten Harnstoffen mit Nitrit in schwachsaurer Lösung kann zu zwei Produkten führen, dem entsprechenden Nitrosoharnstoff und durch Nitrosospaltung dem Dialkylnitrosamin. Ausser bei *N*-Phenyldialkylharnstoffen ist Nitrosoharnstoff das Hauptprodukt. Phenyldialkylharnstoffe und trisubstituierte Thioharnstoffe ergeben Dialkylnitrosamin als das einzige *N*-Nitrosoprodukt. Tetraalkylharnstoffe und bis-(Dialkylthiocarbamyl)disulfide bilden auch Dialkylnitrosamine durch Reaktion mit salpetriger Säure. Die Ausbeute an diesen carcinogenen Nitrosaminen ist bei 37°C und bei niedrigen Konzentrationen des tertiären Amins (0,01 M) signifikant. Das Insektizid, Carbaryl, reagiert mit salpetriger Säure zu einem hochmutagenen *N*-Nitrosoderivat.

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Induction of Liver and Lung Tumours in Rats by the Simultaneous Administration of Sodium Nitrite and Morpholine

P. M. NEWBERNE and R. C. SHANK

Department of Nutrition and Food Science, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139, USA

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Abstract—Long-term feeding studies were conducted in Sprague–Dawley rats using various dietary concentrations (up to 1000 ppm each) of nitrite and morpholine or 5 or 50 ppm *N*-nitrosomorpholine. The animals fed nitrite and morpholine at moderate to high concentrations developed hepatocellular carcinomas and angiosarcomas, histologically identical to those induced by preformed *N*-nitrosomorpholine. The data suggest that *in vivo* nitrosation of morpholine does occur in the rat, presumably in the acidic gastric environment.

INTRODUCTION

The carcinogenicity of preformed nitrosamines has been documented in many animals and species (Magee & Barnes, 1967) and continues to be of interest. However, there is increasing concern about nitrosation of dietary amines by sodium nitrite, a common food additive, and the possible relationship of this reaction to cancer in man (Druckrey, Preussmann, Ivankovic & Schmähl, 1967; *Food and Cosmetics Toxicology*, 1968; *Lancet*, 1968; Lijinsky & Epstein, 1970). Nitrosamines have been found in such varied items as tobacco smoke and mushrooms (*Food and Cosmetics Toxicology*, 1968) and, more significantly, in staple foods such as grains, pasteurized milk and cheese (Hedler & Marquardt, 1968) and nitrite-treated cheese, fish, smoked fish and meat (Ender & Čeh, 1968; *Food and Cosmetics Toxicology*, 1968).

Furthermore, there is the disturbing suggestion that nitrosamines may be formed from nitrite and secondary amines under the acidic conditions of the human stomach (Druckrey, Steinhoff, Beuthner, Schneider & Klärner, 1963; Sander, 1967). Although Sander, Schweinsberg & Menz (1968) had demonstrated the *in vivo* formation of nitrosamines in the stomachs of rats simultaneously fed nitrite and secondary amines of fairly low pH, the interest in this phenomenon was raised considerably when Sander & Seif (1969) showed that patients given nitrite and diphenylamine produced nitrosodiphenylamine in the stomach. These considerations lend an urgency to this area of environmental carcinogenesis paralleled only by the recently recognized mycotoxin hazard (Ciegler, Kadis & Ajl, 1971).

Whether or not biologically significant amounts of nitrosamines are formed in the human stomach from nitrite and the amines normally present in food products is a matter of conjecture. However, research to date strongly suggests the need to learn more about *in vivo* nitrosation and its relation to carcinogenesis. Only a few studies have dealt with the long-term administration of secondary amines and nitrite to intact animals. Druckrey *et al.*

(1963) reported negative results when sodium nitrite and diethylamine were fed to rats, and Sander *et al.* (1968) observed that diethylamine and *N*-methylaniline were also without carcinogenic effect when fed along with nitrite to rats. However, Sander & Burkle (1969) have reported the occurrence of hepatic and oesophageal tumours in rats fed *N*-methylbenzylamine or morpholine along with sodium nitrite. More recently, Greenblatt, Mirvish & So (1971) have presented data which suggest that an increased incidence of lung adenomas in mice resulted from *in vivo* nitrosation of secondary amines by dietary nitrite.

The work reported here provides further evidence that dietary nitrite, as the sodium salt, and a secondary amine (morpholine) can induce liver and lung tumours identical to those induced by *N*-nitrosomorpholine, a preformed nitrosamine.

EXPERIMENTAL

Female Sprague–Dawley rats were fed from conception an agar-gel diet, the composition of which has been reported previously (Wogan & Newberne, 1967). The diet contained either *N*-nitrosomorpholine or nitrite and morpholine at various concentrations including those listed in Table 1. In addition, nitrite and morpholine were each fed separately at 1000 ppm but these are not listed because the results were negative. Following parturition, litters were adjusted to eight each and left with the mother until weaning; they then continued on their respective diets. Some of the F_1 generation were bred to provide F_2 generations for long-term carcinogenic studies.

The diets were freshly prepared each week and were stored in covered containers under refrigeration. Analytical-grade sodium nitrite (J. T. Baker Chemical Co., Phillipsburg, N.J.) and morpholine (purified, Fisher Scientific Co., Fairlawn, N.J.) were mixed into the diet to provide the various desired concentrations. *N*-Nitrosomorpholine (Schuchardt GmbH, Munich, Federal Republic of Germany) was fed as a positive control at a dietary level of 5 or 50 ppm (dry weight). All animals were observed at least twice daily and were weighed weekly, and complete autopsies were performed at death or when the rats were moribund. Routine histopathology studies were conducted on all animals.

RESULTS

Significant differences in tumour incidence were not observed between sexes or between generations at the highest concentrations of nitrite and morpholine; for this reason, results from both sexes and both generations were combined for each of the groups fed the high levels of nitrite and morpholine and for those fed the two levels of *N*-nitrosomorpholine, and these combined data are listed as single treatment groups in Table 1. Of the 159 rats fed a diet containing 1000 ppm sodium nitrite and 1000 ppm morpholine, almost 100% (156/159) developed hepatocellular carcinoma and about 25% of these had coexisting hepatic angiosarcomas, which appeared to derive from sinusoidal endothelium or from Kupffer cells. More than 68% of the liver-cell tumours metastasized to the lung, where primary angiosarcomas were also present in about 25% of the cases.

When the concentration of morpholine was decreased to 50 ppm, there was a concomitant drop in the incidence of all tumours and there were fewer metastases of liver tumours to the lung. When morpholine was decreased to 5 ppm, only 3/160 animals developed liver-cell carcinoma even though the nitrite intake remained high (1000 ppm). None of these animals had detectable metastatic lesions, nor were angiosarcomas observed in any of them.

Table 1. *Tumours in rats exposed to continuous dietary* N-*nitrosomorpholine or nitrite and morpholine*

Additions to diet (ppm)			Tumour incidence*			
			Liver		Lung	
$NaNO_2$	Morpholine	*N*-Nitrosomorpholine	Hepatocellular carcinoma	Angiosarcoma	Metastatic hepatocellular carcinoma	Angiosarcoma
0	0	0	0/169	0/169	0/169	0/169
1000	1000	0	156/159	38/159	109/159	37/159
1000	50	0	24/122	11/122	10/122	2/122
1000	5	0	3/160	0/160	0/160	0/160
50	1000	0	4/120	0/120	0/120	0/120
0	0	5	17/132	1/132	1/132	0/132
0	0	50	77/97	10/97	21/97	4/97

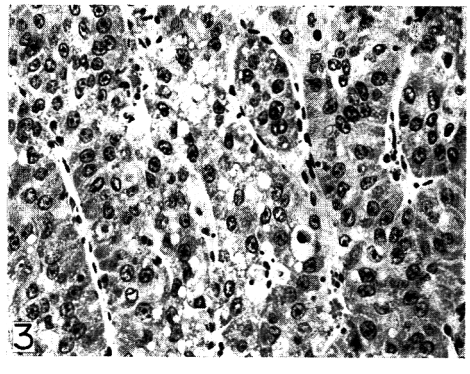
*No. of rats affected/no. observed.

FIG. 1. Liver tumours with peritoneal visceral extension. The closed arrow indicates a nodule subsequently confirmed as a hepatocellular carcinoma. The open arrow indicates a tumour which histological examination revealed as a haematoma with a surrounding angiosarcomatous component.

FIG. 2. Multicentric liver tumour typical of those induced by monocrotaline, aflatoxin and acetylaminofluorene and now observed both with *N*-nitrosomorpholine and with nitrite and morpholine. This type of tumour was more common with the lower level of *N*-nitrosomorpholine or when either nitrite or morpholine was decreased from the 1000 ppm concentration.

FIG. 3. Section taken from the nodule indicated by the closed arrow in Fig. 1. The histological arrangement is typical of the trabecular-type liver-cell carcinoma. Haematoxylin and eosin × 190.

FIG. 4. Section taken from an anaplastic nodule in the mesenteric extension of Fig. 1. The rapidly growing area is characteristic of a large proportion of nitrite–morpholine induced liver-cell tumours. Haematoxylin and eosin × 410.



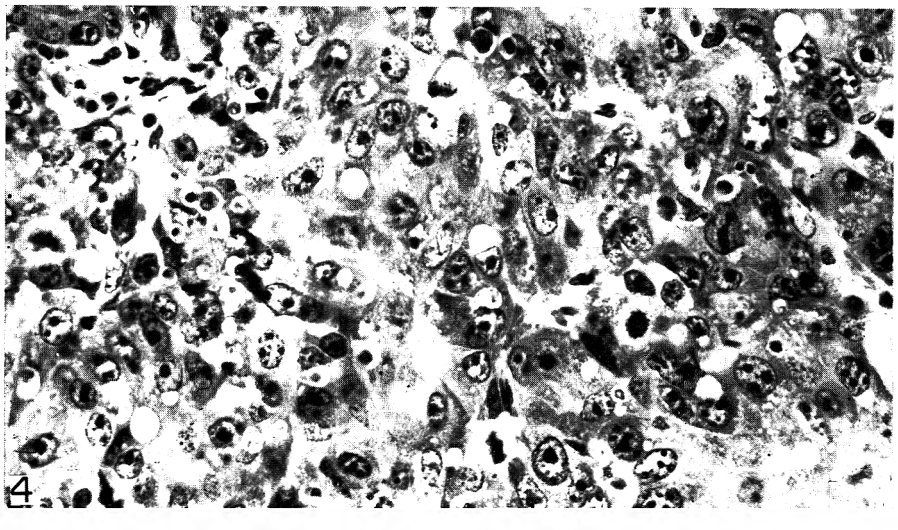


FIG. 5. Highly vascular area of a liver tumour from a rat fed nitrite and morpholine continuously for 33 wk This section was adjacent to the haematoma described in Fig. 1. Haematoxylin and eosin × 80.

FIG. 6. A typical haemangiomatous area, adjacent to the section shown in Fig. 5. The cavernous vascular space is lined by cells (closed arrows), arranged in solid masses or in linear fashion, sometimes forming tubular structures, which contained erythrocytes. Haematoxylin and eosin × 80.

FIG. 7. An area of solid tumours, firm and glistening on gross observation, adjacent to a large vascular tumour similar to that shown in Fig. 6. The cells varied in morphological form but tended to be hyperchromatic, spindle-shaped and more vascular than adjacent tissues. Mitotic figures were abundant in tumours of this type, which were assessed as angiosarcomas. Haematoxylin and eosin × 100.

FIG. 8. Gross appearance of metastatic lesions of liver tumours to lung (arrowed). The large number of metastatic lesions, numbering up to 27/lobe, indicated a fast-growing, early metastasizing liver tumour.

FIG. 9. A characteristic liver-cell tumour, which had metastasized to the lung. The cells are typical of the hepatocellular carcinoma resident in the rat from which this lung section was taken. Haematoxylin and eosin × 190.

FIG. 10. Left portion (arrowed) indicates a section of an angiosarcoma in the lung of a rat with a coexisting liver-cell carcinoma. The lesion on the right (arrowed) indicates a carcinoma metastatic from the liver. Haematoxylin and eosin × 32.

FIG. 11. High-power view of the section taken from the lesion on the left of Fig. 10. The cells are pleomorphic, bizarre and hyperchromatic, typical of angiosarcomas observed in these studies. Haematoxylin and eosin × 380.

FIG. 12. High-power view of the section taken from lesion on the right of Fig. 10, showing a typical anaplastic hepatocellular carcinoma with numerous mitotic figures. Haematoxylin and eosin × 425.

Decreasing the nitrite concentration to 50 ppm and maintaining the morpholine level at 1000 ppm resulted in liver-cell carcinomas in 4/120 animals. Studies on both the group given the high level of *N*-nitrosomorpholine (50 ppm) and that given the high level of nitrite and morpholine (1000 ppm of each) are completed, so the statistics on these groups are firm. Results of the other concentrations may be modified slightly when all the animals have been accounted for, but the trends are clear and the high incidence and rapid induction of tumours with nitrite and morpholine imply a more complex system than is indicated by kinetic studies.

The tumours induced by *N*-nitrosomorpholine and by nitrite and morpholine were morphologically identical. Grossly, most livers presented massive, multicentric nodular tumours with a remarkable vascular component, often with extensive haemorrhage or large haematomas (Fig. 1). There was extensive destruction of the liver by greyish-white nodules and dark blood-filled cavities; rapid extension throughout the abdominal cavity was usual. In all groups, however, some rats had smaller liver tumours (Fig. 2) resembling those induced by other hepatocarcinogens, including aflatoxin, acetylaminofluorene and monocrotaline. The rapidly growing soft tumorous nodules, away from frankly necrotic or haemorrhagic areas, were histologically either trabecular (Fig. 3) or anaplastic (Fig. 4), with the latter predominating.

Highly vascular areas consisting of blood-filled spaces (Fig. 5) lined either by neoplastic liver cells or by sarcomatous cells derived from the endothelium (Fig. 6) were present in many of the tumours. In some cases, rupture of blood-filled spaces into the peritoneal cavity resulted in death from haemorrhage. In many tumour-bearing rats, somewhat firm, solid angiosarcomas (Fig. 7) coexisted with liver cell carcinomas and both types of tumours metastasized to the lung (Figs 8–12). Since primary angiosarcomas also arose in the lungs of some animals, it was not always possible to determine whether the sarcomas were primary to the lung or were metastatic from the liver. Generally, the enormous size of the liver and the rapidity and marked degree of peritoneal extension and lung metastasis exceeded that observed with any of several standard liver carcinogens studied in our laboratories. The rapid extension and metastasis may have been related to the vascularity of the tumours, but this is speculative.

DISCUSSION

These data support the concept that *in vivo* nitrosation does occur, presumably under the acidic conditions in the rat's stomach. It is quite likely, however, that nitrosation occurs in some other biological compartment, or that some other modification takes place which results in chemical conversion favourable to carcinogenesis. The original concepts of Sander (1967) and Lijinsky & Epstein (1970) are extremely provocative, but we must bear in mind that nitrates and nitrites, as well as amines, have been abundant in our environment for many years without an appreciable increase in chemically related cancer of the types produced in experimental animals. We must therefore proceed with caution in attempting to extrapolate to man the results obtained in animals.

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REFERENCES

Ciegler, A., Kadis, S & Ajl, S. (1971). *Microbial Toxins. A Comprehensive Treatise. Vol. VI. Fungal Toxins.* Academic Press, New York.

Druckrey, H., Preussmann, R., Ivankovic, S. u. Schmähl, D. (1967). Organotrope carcinogene Wirkungen bei 65 verschiedenen N-Nitroso-Verbindungen an BD-Ratten. *Z. Krebsforsch.* **69,** 103.

Druckrey, H., Steinhoff, D., Beuthner, H., Schneider, H. u. Klärner, P. (1963). Prüfung von Nitrit auf chronisch toxische Wirkung an Ratten. *Arzneimittel-Forsch.* **13,** 320.

Ender, F. & Čeh, L. 1968). Occurrence of nitrosamines in foodstuffs for human and animal consumption. *Fd Cosmet. Toxico* . **6,** 569.

Food and Cosmetics Toxicology (1968). Nitrosamines: A jig-saw puzzle with missing pieces. *ibid* **6,** 647.

Greenblatt, M., Mirvish, S. & So, B. T. (1971). Nitrosamine studies: Induction of lung adenomas by concurrent administration of sodium nitrite and secondary amines in Swiss mice. *J. natn. Cancer Inst.* **46,** 1029.

Hedler, Liselotte & Marquardt, P. (1968). Occurrence of diethylnitrosamine in some samples of food. *Fd Cosmet. Toxicol.* **6** 341.

Lancet (1968). Nitrites, nitrosamines, and cancer. *ibid* **i,** 1071.

Lijinsky, W. & Epstein, S. S. (1970). Nitrosamines as environmental carcinogens. *Nature, Lond.* **225,** 21.

Magee, P. N. & Barnes, J. M. (1967). Carcinogenic nitroso compounds. *Adv. Cancer Res.* **10,** 163.

Sander, J. (1967). Kann Nitrit in der menschlichen Nahrung Ursache einer Krebsentstehung durch Nitrosaminbildung sein? *Arch. Hyg. Bakt.* **151,** 22.

Sander, J. u. Bürkle, G. (1969). Induktion maligner Tumoren bei Ratten durch gleichzeitige Verfütterung von Nitrit und sekundären Aminen. *Z. Krebsforsch.* **73,** 54.

Sander, J., Schweinsberg, F. u. Menz, H.-P. (1968). Untersuchungen über die Entstehung cancerogener Nitrosamine im Magen. *Hoppe-Seyler's Z. physiol. Chem.* **349,** 1691.

Sander, J. u. Seif, F. (1969). Bakterielle Reduktion von Nitrat im Magen des Menschen als Ursache einer Nitrosamin-Bildung. *Arzneimittel-Forsch.* **19,** 1091.

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

Induction de tumeurs hépatiques et pulmonaires chez le rat par l'administration simultanée de nitrite de sodium et de morpholine

Résumé—Dans des essais nutritionnels de longue durée, on a fait prendre à des rats Sprague–Dawley soit différentes doses (jusqu'à 1000 ppm du régime) de nitrite et de morpholine, soit 5 ou 50 ppm de *N*-nitrosomorpholine. Les animaux qui recevaient le nitrite et la morpholine à des doses modérées ou élevées ont contracté des carcinomes et des angiosarcomes hépatocellulaires histologiquement identiques à ceux provoqués par la *N*-nitrosomorpholine préformée. Les données suggèrent que la nitrosation *in vivo* de la morpholine chez le rat se produit probablement dans le milieu acide de l'estomac.

Induktion von Leber- und Lungentumoren bei Ratten durch die gleichzeitige Anwendung von Natriumnitrit und Morpholin

Zusammenfassung—Langzeitverfütterungsversuche wurden mit Sprague–Dawley-Ratten unter Anwendung verschiedener Konzentrationen (bis 1000 ppm jeder Verbindung) von Nitrit und Morpholin oder 5 oder 50 ppm *N*-Nitrosomorpholin durchgeführt. Die Tiere, welche Nitrit und Morpholin in mässigen bis hohen Konzentrationen im Futter erhielten, entwickelten hepatocelluläre Carcinome und Angiosarcome, histologisch identisch mit den von ausserhalb des Körpers gebildetem *N*-Nitrosomorpholin induzierten. Die Daten lassen annehmen, dass *in-vivo*-Nitrosation von Morpholin in der Ratte vorkommt, vermutlich innerhalb des sauren Mageninhalts.

Fd Cosmet. Toxicol. Vol. 11, pp. 827–840. Pergamon Press 1973. Printed in Great Britain

Dietary Copper and the Induction of Neoplasms in the Rat by Acetylaminofluorene and Dimethylnitrosamine

W. W. CARLTON and PEGGY S. PRICE

Department of Veterinary Microbiology and Pathology, School of Veterinary Science and Medicine, Purdue University, Lafayette, Indiana 47907, USA

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Abstract—The effect of dietary copper on the incidence and location of neoplasms was studied in rats fed copper-deficient (1 ppm) and excess-copper (800 ppm) diets containing acetylaminofluorene (AAF) and in rats fed the same copper-deficient and excess-copper diets and given dimethylnitrosamine (DMN) in the drinking-water for 9 months. The excess copper with or without DMN or AAF was toxic, body-weight gains being reduced and mortality increased. Reduction in weight gain was greatest in the excess-copper–AAF group, but the highest mortality (72%) occurred in the excess-copper–DMN group. Liver weights were increased in the AAF-treated rats, partly because of the presence of neoplasms. The average copper content (4·5 ppm) in hepatic tissue from rats fed the copper-deficient diet was greater than that found in hepatic tissue from DMN-treated (3·9 ppm) or AAF-fed rats (2·8 ppm). Hepatic copper concentrations of rats fed the excess-copper diet averaged 244 ppm in controls, while the comparable figures for the DMN-treated and AAF-fed rats were 394 and 354 ppm respectively. Neoplastic hepatic tissue from rats fed the copper-deficient diet had a copper content similar to that of the non-neoplastic tissue, but the neoplastic hepatic tissue from rats fed the excess-copper diet was lower than that of non-neoplastic liver. Neoplastic tissue from the kidney had less copper than grossly normal renal tissue. The incidence of hepatic neoplasms was similar in DMN-treated rats fed either the copper-deficient or excess-copper diets, but kidney neoplasms occurred in 57% of the rats receiving the copper-deficient–DMN treatment and killed for autopsy, compared to an incidence of 0·0% in the excess-copper–DMN group. This difference in incidence may be related to the copper levels of the diet. The incidence of DMN-induced neoplasms in the lungs was similar in rats fed the copper-deficient and excess-copper diets. More AAF-fed than DMN-treated animals developed hepatic neoplasms, but dietary copper concentration did not have any effect on the incidence. Extrahepatic neoplasms in AAF-fed rats occurred in the lungs, spleen, skin, intestine, pancreas and muscle. The incidence of extrahepatic neoplasms was 40% in copper-deficient–AAF rats killed for autopsy, but only 17% in those fed the excess-copper diet.

INTRODUCTION

In published studies on the relationship between the concentration of dietary copper and the induction of neoplasia by chemical carcinogens, the results have been variable. Thus, Pedrero & Kozelka (1951) found that the incidence of hepatic tumours induced by dimethylaminoazobenzene (DMAB) was 86·3% in rats fed the carcinogen alone and 66·6% in rats fed the carcinogen and 0·25% copper sulphate. King, Spain & Clayton (1957) found that the number of hepatic neoplasms in rats fed DMAB and a diet high in copper (300 ppm) was lower than that observed in rats fed a normal copper level (4 ppm). Howell (1958) concluded that copper oxyacetate had a strong retarding effect on hepatoma development in rats treated with DMAB. Fare & Howell (1964) investigated the effect of dietary copper on the carcinogenic effect of two dyes, 3-methoxy-4-aminoazobenzene and 3-methoxy-4-monomethylaminoazobenzene, observing hepatic neoplasms in both unsupplemented dye groups but no hepatic tumours in rats fed the carcinogenic diets containing 0·5% copper oxyacetate.

In contrast to the above reports, Goodall (1964) found that the addition of copper to the drinking-water did not prevent the induction of hepatomas in rats painted with a solution of 2-aminofluorene. Dietary modification of the carcinogenic response to chemicals has achieved much significance with the reports of the induction of neoplasms by the concurrent administration of sodium nitrite and secondary amines (Greenblatt, Mirvish & So, 1971; Sander & Burkle, 1969) and the demonstration of the formation of dimethylnitrosamine (DMN) from sodium nitrite and dimethylamine by bacterial flora of the rat intestine (Klubes & Jondorf, 1971). A study was undertaken to ascertain whether a high level of copper would have an inhibitory effect on the induction of neoplasia by acetylaminofluorene (AAF) or DMN and to determine whether the incidence of neoplasia would be increased or whether neoplasms would appear earlier in rats fed a diet low in copper. The results of this study form the basis of this report.

EXPERIMENTAL

Animals, diets and treatment

Male Sprague–Dawley rats obtained from a commercial supplier were placed on the test diets soon after weaning. They were housed in wire-bottom stainless-steel cages and were given feed and water *ad lib.* Fresh feed was provided daily. The rats were weighed weekly and observations were made daily as to their general health.

The purified basal diet (copper-deficient) was purchased from a commercial source and was prepared for copper-deficiency studies in the rat (Mills & Murray, 1960). The diet was analysed by an independent laboratory (WARF) using atomic absorption spectrophotometry and was found to contain 1 ppm copper. Six experimental groups containing 50–102 rats were included in this study. Three groups were fed the copper-deficient diet and three groups received this basal diet with supplemental copper (excess-copper diet) added in the form of cupric sulphate to give a copper concentration of 800 ppm. Within each of these two dietary regimens, one group received DMN in the drinking-water and one group was fed AAF in the diet. Groups without the carcinogens served as controls. The initial numbers of rats in each group varied because preliminary studies had shown that some treatments were more toxic than others and it was hoped by this means to ensure an adequate level of survival in each group for 7 or 8 months of treatment.

DMN was added to the drinking-water at a concentration of 50 ppm and the solution was given for 4 days followed by 4 days on distilled water. This regimen was followed because preliminary studies established that higher concentrations of DMN were too toxic. Administration of the carcinogenic solution was discontinued after 6 months.

AAF was added to the diets at a concentration of 0·06%. These test diets were fed for 4 days followed by the feeding of the copper-deficient or excess-copper diets for 4 days and this procedure was continued for the rest of the 6-month period.

Post-mortem studies

After 90 days of feeding, five rats from each diet group were killed. Thereafter, each month (30 days) an additional five animals from each group were killed. Rats were generally selected at random.

Animals were killed by decapitation and exsanguination. The spleen, kidneys, lungs, heart, thyroid gland, adrenal glands, duodenum and pancreas were taken from each animal

and placed in 10% formalin. The liver was divided into two portions; one was placed in a glass container for freezing prior to chemical analysis for copper content, and the other portion was fixed in formalin. Only enlarged neoplastic kidneys were weighed prior to fixation. Fixed tissues were trimmed and processed for the preparation of paraffin sections and were stained with haematoxylin and eosin for histopathological examination.

Concentrations of copper in non-neoplastic and neoplastic hepatic and renal tissues were determined by atomic absorption spectrophotometry. The procedure used for the preparation of the tissues was similar to that described by Parker, Humoller & Mahler (1967). Copper analyses were carried out on 5 g samples of liver obtained at the monthly periods from each of the six diet groups. These samples were made up of approximately 1 g of tissue from each of the five rats. The analyses were run in triplicate and precautions were taken to prevent copper-contamination of the tissues, which were collected using stainless-steel equipment and acid-washed glassware rinsed in glass-distilled water.

RESULTS

Growth response

Mean data on body weights are summarized in Fig. 1. Rats fed the copper-deficient control diet had the highest mean body weights of the various groups. The mean weights of the copper-deficient–DMN group were well below those of the copper-deficient control rats. The excess-copper control and excess-copper–DMN groups had similar mean weights approximately 120 g below the mean weight of the copper-deficient control group after

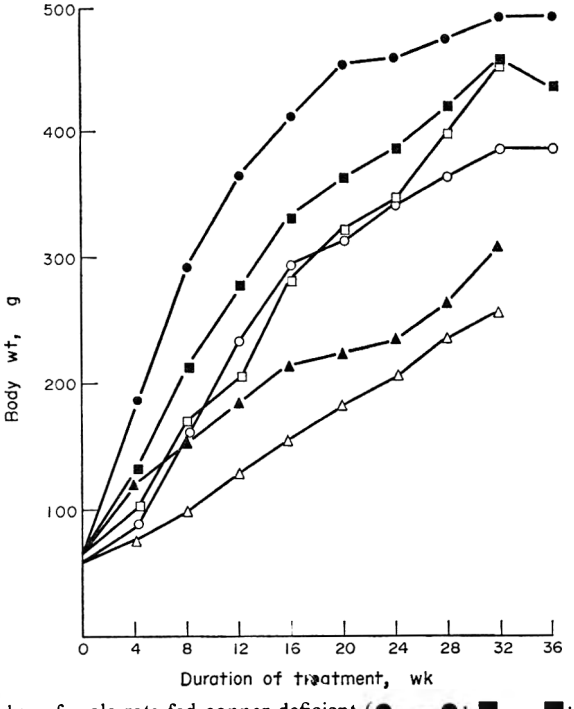

FIG. 1. Mean body weights of male rats fed copper-deficient (●——●; ■——■; ▲——▲) and excess-copper (○——○; □——□; △——△) diets and treated with DMN (■; □), AAF (▲; △) or no carcinogen (controls: ●; ○).

6 months of feeding. AAF was markedly toxic and the mean weights of rats fed either of the AAF diets were markedly below those of the copper-deficient control, with the excess-copper–AAF group having the lowest mean weights of the various groups.

Mortality

By the end of 3 months of feeding, the mortality rate in the six groups varied from 2 to 69% (Table 1). Deaths were least in the copper-deficient control and greatest in the excess-

Table 1. *Mortality in groups of rats fed copper-deficient or excess-copper diets with DMN or AAF treatment for 3–9 months*

Duration of treatment (months)	Diet...	Copper-deficient			Excess-copper		
	Other treatment...	Control	DMN	AAF	Control	DMN	AAF
	No. of rats*...	50	74	55	58	102	65
		Mortality (%)					
3		2	38	15	33	69	39
4		2	41	15	40	71	39
5		8	45	20	43	71	40
6		10	49	35	43	71	40
7		16	53	48	45	72	43
8		16	53	51	45	72	51
9		16	57	—	45	—	54
	Change in % mortality over 6-month period...	14	19	36	12	3	15

DMN—50 ppm dimethylnitrosamine in drinking-water for 4-day periods alternating with distilled water.
AAF—0·06% acetylaminofluorene in the diet for 4-day periods alternating with control diet.
*Total no. of rats started on diet.

copper–DMN group. These data indicate that excess copper, DMN and AAF were all toxic and increased the number of deaths over the number in the copper-deficient control group. At the termination of the experiment, the lowest mortality (16%) was observed in the copper-deficient control groups and the highest (72%) was in the group of rats fed the excess-copper–DMN diet. This group, which had the highest mortality during the first 3 months (69%), had the fewest deaths later.

Organ weights

Liver weights expressed as a percentage of body weight (Table 2) were very similar for all groups except those receiving AAF.

Between the two latter groups, the copper-deficient–AAF group generally had higher mean values. Some of the increase in size was due to the presence of neoplasms.

Copper determinations

The copper content of grossly non-neoplastic hepatic tissue from rats fed the copper-deficient diets did not vary greatly, but values for the group fed AAF were generally lower than for the other two groups (Table 3). The copper content of livers from the rats fed the

Table 2. *Liver weight of rats fed copper-deficient or excess-copper diet with DMN or AAF treatment and killed for autopsy after treatment for 3–9 months*

Experimental group	Mean liver weight (% of body weight)*		
Month...	3	5	7
Copper-deficient diet:			
Control	3·2 (3·0–3·6)	2·9 (2·7–3·0)	3·1 (2·8–3·4)
+DMN	3·6 (3·2–4·2)	3·6 (1·3–3·8)	3·2 (2·7–3·1)
+AAF	6·3 (4·8–8·5)	16·2 (13·4–18·8)†	12·8 (9·4–16·1)†
Excess-copper diet:			
Control	4·0 (3·5–4·9)	3·3 (2·9–3·8)	3·3 (3·0–3·5)
+DMN	4·1 (2·9–4·7)	3·7 (2·7–5·1)	3·7 (3·1–4·3)
+AAF	5·9 (5·2–7·0)	6·4 (5·5–7·2)†	9·3 (6·4–12·1)†

DMN—50 ppm dimethylnitrosamine in drinking-water for 4-day periods alternating with distilled water.
AAF—0·06% acetylaminofluorene in the diet for 4-day periods alternating with control diet.
*With ranges of values in parentheses.
†Large liver weights were due to the presence of hepatomas or hepatocellular carcinomas.

Table 3. *Copper content of non-neoplastic liver tissue from rats fed copper-deficient and excess-copper diets with DMN or AAF treatment for 3–8 months*

Duration of treatment (months)	Copper content (ppm)*					
Diet...	Copper-deficient			Excess-copper		
Other treatment...	Control	DMN	AAF	Control	DMN	AAF
3	4·6	3·2	3·1	314	380	412
4	4·2	3·5	2·9	234	460	372
5	4·3	4·5	2·0	236	432	418
6	4·1	4·4	2·2	200	270	312
7	5·0	4·2	2·6	236	383	314
8	4·8	3·8	3·9	—	438	294
Mean...	4·5	3·9	2·8	244	394	354

DMN—50 ppm dimethylnitrosamine in drinking-water for 4-day periods alternating with distilled water.
AAF—0·06% acetylaminofluorene in the diet for 4-day periods alternating with control diet.
*One pooled sample was analysed for each group at each time.

excess-copper diets with carcinogens was greater than that found in the excess-copper control rats, but no other consistent pattern emerged, although the mean of all determinations was greatest for the group receiving DMN.

The copper content of neoplastic hepatic tissue from rats receiving copper-deficient carcinogenic diets was similar to that found in grossly normal tissue (Table 4). In the two groups of rats which were fed the excess-copper–AAF diet and had grossly separable neoplasms, the neoplastic tissue contained less copper than the non-neoplastic tissue from the same animal.

Renal neoplasms were observed grossly only in the copper-deficient–DMN rats and the concentrations of copper were lower in these neoplasms than in non-neoplastic renal tissue.

Table 4. *Copper content of selected hepatic neoplasms compared with that of non-neoplastic hepatic tissue from rats fed copper-deficient and excess-copper diets with DMN and AAF treatment*

Experimental group	Duration of treatment (months)	Copper content (ppm) of Non-neoplastic tissue	Copper content (ppm) of Neoplastic tissue
Copper-deficient diet:			
+DMN	4	3·5	4·2
	6	4·4	4·4
+AAF	5	2·0	1·9
	6	2·2	2·6
	7	2·6	2·6
	8	3·9	2·7
Excess-copper diet:			
+AAF	5	418	347
	8	294	163

DMN—50 ppm dimethylnitrosamine in drinking-water for 4-day periods alternating with distilled water.
AAF—0·06% acetylaminofluorene in the diet for 4-day periods alternating with control diet.

The copper concentration of this latter tissue was somewhat lower than that found in kidneys from the copper-deficient control rats (Table 5). The copper content of large neoplasms was somewhat lower than the concentration found in small neoplasms (under 22 g).

Table 5. *Copper content of selected renal samples from rats fed a copper-deficient diet (1 ppm) with or without DMN treatment*

Experimental group	Tissue	Copper content (ppm) of renal tissues at month 5	6	7	8
Copper-deficient diet:					
Control	Normal	8·1	7·6	9·3	9·4
+DMN	Non-neoplastic	7·3	7·0	7·2	7·1
	Small neoplasms	5·5	2·6	2·7	—
	Large (>22 g) neoplasms	2·4	2·0	1·8	2·0

DMN—50 ppm dimethylnitrosamine in drinking-water for 4-day periods alternating with distilled water.

Gross lesions

Livers from rats fed the copper-deficient–DMN diet for 3 or 4 months varied in appearance from those that were grossly normal to ones with severe macroscopic alterations and complete fusion of lobes. Some livers were tan-coloured and slightly swollen. Livers from rats fed this diet for 5–8 months presented abnormal features in varying degrees of severity: swelling, variation in colour, presence of clear cysts, haematocysts and/or neoplasms.

Grossly visible lesions of the livers were observed at the various monthly samplings in rats fed the copper-deficient–AAF diet. The abnormalities observed after 3 months of

feeding included discoloration, enlargement and presence of focal pale areas. After 4 months of feeding a few clear cysts were also present. Later, the livers were pale, cystic and markedly enlarged, and neoplasms ranging in size from pin-point nodules to masses measuring up to 2 cm were observed in all lobes (Fig. 2).

The livers from copper-deficient and excess-copper control rats were grossly normal. Livers from rats receiving the DMN water and the excess-copper diet were either normal or slightly off-colour when examined after the rats had been fed for 3 and 4 months. Few further changes were observed after 5 and 6 months except for prominent capsular vessels. Cysts, swollen lobes and haematocysts occurred in the livers of rats fed for 7 months. Livers from the four rats killed after 8 months on this diet were more severely affected; haematocysts were observed in two livers and a neoplasm was found in one other.

Livers of rats treated with the excess-copper–AAF diet had a striking gross appearance which was obvious in one rat at month 3 and was consistently present in one or more livers at the other autopsy periods. The hepatic surface was converted into a mass of small nodules resembling cobblestones (Fig. 3). This nodularity was always more marked on the visceral surface. In addition to the nodular capsular surface, clear cysts were present peripherally after 5 months. Increased hepatic size, cysts and small white foci were additional abnormalities appearing after 6 months of feeding. Neoplasms, when present, were larger after 7 months and all livers from rats fed for 8 months presented clear cysts, neoplasms and capsular nodularity, but there was still some variation in the severity of the gross alterations (Fig. 4)

Grossly enlarged kidneys with neoplasms were first observed after 5 months in rats receiving the copper-deficient–DMN treatment (Fig. 5). The kidneys of four of the five rats had neoplasms varying in diameter from 1 to 5 cm. After 6 months, neoplasms of the kidneys were present in all five rats. Grossly apparent neoplasms were present in three of the five rats examined after 7 months of feeding and one was especially large, weighing 89·6 g (Fig. 6). Only one renal neoplasm was obvious at autopsy in five rats killed after 8 months. Three of 13 rats which received this treatment and which died during the experimental period had grossly apparent renal neoplasms.

Abnormalities other than alterations of the liver and kidneys observed at autopsy in rats of the copper-deficient–DMN group included pale, expanding masses in the lungs of two rats. Grossly detectable neoplasms were observed in the lungs of excess-copper–DMN rats after 7 and 8 months of feeding.

Neoplasms at locations other than the liver were most numerous in the rats fed the copper-deficient–AAF diet. Thus, after 5 months, three rats had grossly obvious neoplasms in one or more of the following locations: ventral throat area, middle of side, groin area and base of ear. After 6 months, neoplasms were noted in the lungs from two rats and in the spleen of another. At autopsy at month 7, neoplasms were present in such locations as the ventral thorax, spleen, abdomen, perianal region, base of ear, the right rear leg and the small intestine.

Far fewer extrahepatic neoplasms were found in rats fed the excess-copper–AAF diet. Those that occurred were located at the base of the ear, along the lateral abdomen and in the lungs.

No gross abnormalities were observed in the urinary bladder of animals in any group.

Histopathological lesions

Non-neoplastic lesions of common occurrence in the livers of carcinogen-treated rats

included biliary-ductule cell hyperplasia, proliferation of biliary ducts and the presence of haematocysts. Many of the proliferated biliary ducts were dilated and some were markedly enlarged, accounting for the clear cysts noted at autopsy. The cystic ducts had an epithelial lining of simple squamous to low cuboidal cells and when multiple were separated by a fine connective-tissue stroma.

The incidence rates of hepatic neoplasms and other lesions observed in the rats fed the various diets are summarized in Table 6. Lesions listed as transitional nodules were localized groups of hepatocytes differing in staining intensity from the surrounding parenchyma but showing only minimal deviation of nuclear morphology and no compression of the surrounding parenchyma, those classed as hepatomas were larger foci of hepatocytes showing changes in nuclear morphology and causing compression of the surrounding parenchyma, and the hepatocellular carcinomas were large, highly cellular neoplasms showing marked alterations in nuclear and cytoplasmic morphology, containing areas of necrosis and blood cysts and invading blood and lymph vessels. In addition to hepatomas and hepatocellular carcinomas (Fig. 7), a fibrosarcoma (Fig. 8) and cholangiocarcinoma of the liver were observed in rats of the copper-deficient–DMN group. Hepatomas, hepatocellular carcinomas, cholangiomas (Fig. 9) and one cholangiocarcinoma were observed in the livers of rats fed the copper-deficient–AAF diet. Lung metastases of hepatocellular carcinomas were observed in the AAF-fed groups (Fig. 10). The copper level of the diet appeared to have little or no effect on the incidence rate of hepatic neoplasms.

Fibrosarcomas (Fig. 11), adenomas (Fig. 12) and adenocarcinomas (Fig. 13) were observed in kidneys of rats in the copper-deficient–DMN group. Emboli of tumour cells from a renal fibrosarcoma were observed in the lung (Fig. 14). One fibrosarcoma was found in a kidney from a rat fed the copper-deficient control diet. No renal neoplasms were observed either grossly or microscopically in the rats from other groups killed for autopsy. One renal adenoma was observed in a rat that died after 7 months on the excess-copper–DMN treatment; this was the only renal neoplasm found in 32 rats receiving this treatment.

Neoplasms arising in locations other than the liver and kidneys included primary neoplasms of the lung, spleen, skin and intestine. The neoplasms observed included adnexal gland adenocarcinomas, keratoacanthomas, splenic lymphoma, alveolar-cell adenomas (Fig. 15) and adenocarcinomas, adenocarcinoma arising from the epithelium of the intestinal mucosa, squamous cell carcinomas of the skin and lungs, fibrosarcoma of the dermis and a rhabdomyosarcoma. The incidence rates of these neoplasms appeared to be less in the rats receiving excess copper and a carcinogen (Table 6).

DISCUSSION

The copper-deficient diet (1 ppm) was adequate to sustain normal growth according to the data of Boyden, Potter & Elvehjem (1938) and others (Ross & Bras, 1965; Wolff, 1960). However, the excess-copper diet proved toxic. This contrasts with the findings of several investigators (Fare & Howell, 1964; Fare & Woodhouse, 1963; Howell, 1958; Pedrero & Kozelka, 1951), who fed greater concentrations to rats over extended periods without adverse effect. The age and body weight of the rats when they were placed on the copper-supplemented diets probably accounts for some of this difference. The possibility exists that copper supplementation of a given concentration is more toxic when combined with a purified diet, as in the present study, than when the same level of copper is combined with natural feedstuffs.

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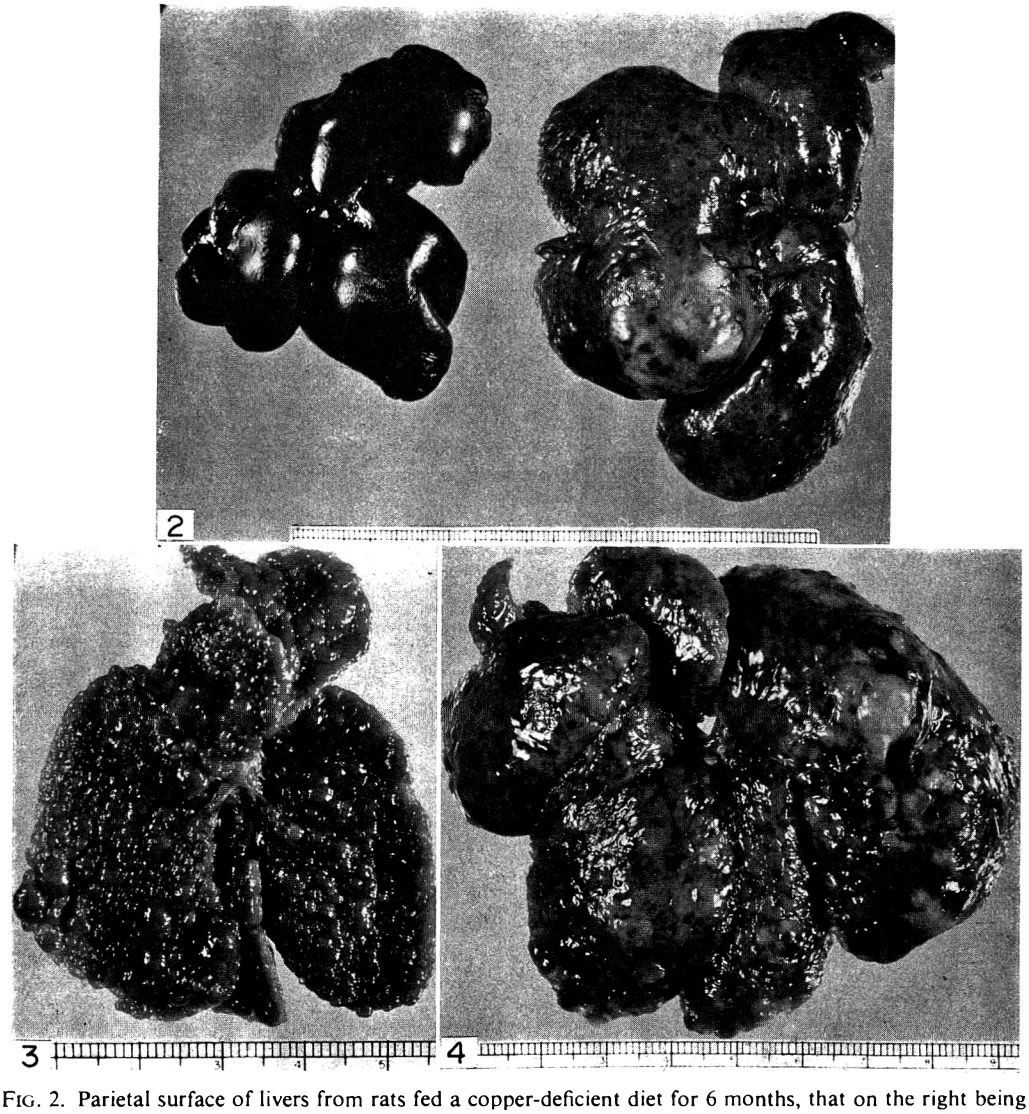


FIG. 2. Parietal surface of livers from rats fed a copper-deficient diet for 6 months, that on the right being from an AAF-treated rat showing numerous clear and blood-filled cysts as well as neoplasms of various sizes. Weight of this liver was 19·7% of body weight.

FIG. 3. Visceral surface of liver from a rat fed the excess-copper–AAF diet for 7 months, showing extensive nodule formation.

FIG. 4. Parietal surface of liver from a rat fed the excess-copper–AAF diet for 8 months, showing numerous clear and haemorrhagic cysts and multiple neoplastic growths in all lobes. Liver weighed 44·4 g, 18·1% of body weight.

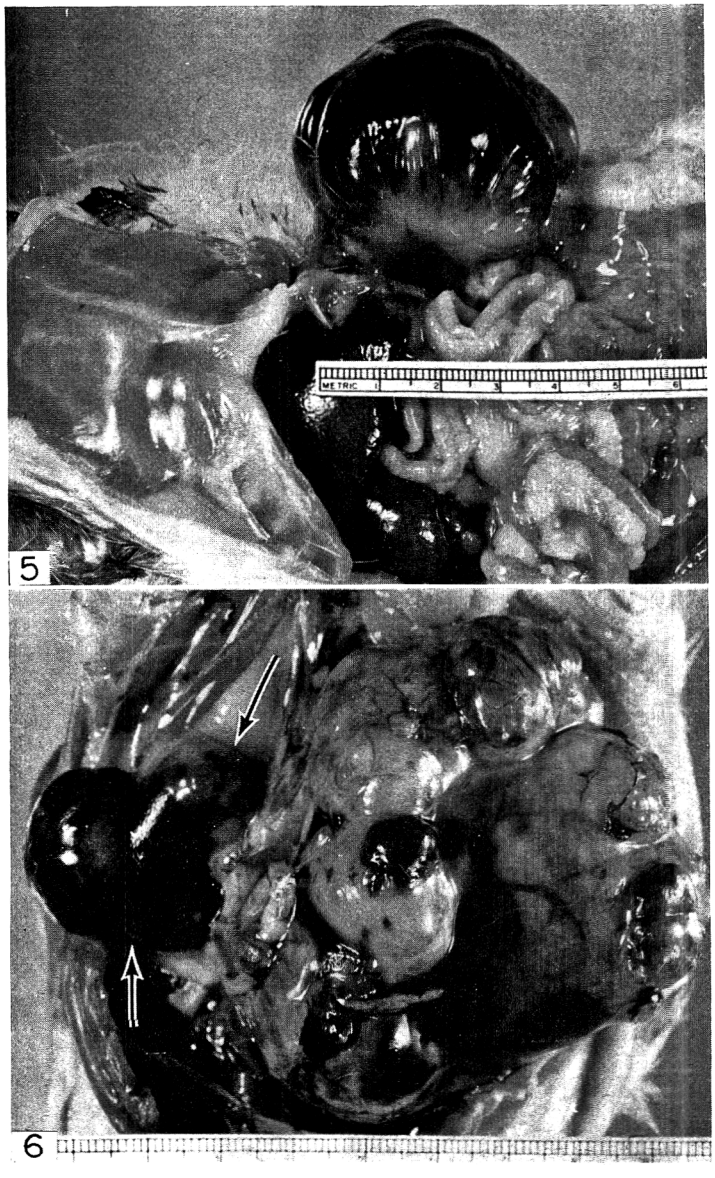


FIG. 5. Neoplasm of left kidney in a rat given the copper-deficient–DMN treatment for 5 months. The kidney with neoplasm weighed 48 g.

FIG. 6. Neoplastic kidneys of a rat fed the copper-deficient–DMN treatment for 7 months. The right kidney was largely replaced by a fibrosarcoma and weighed 89·6 g, and the left kidney presented two grossly apparent neoplasms (arrowed).

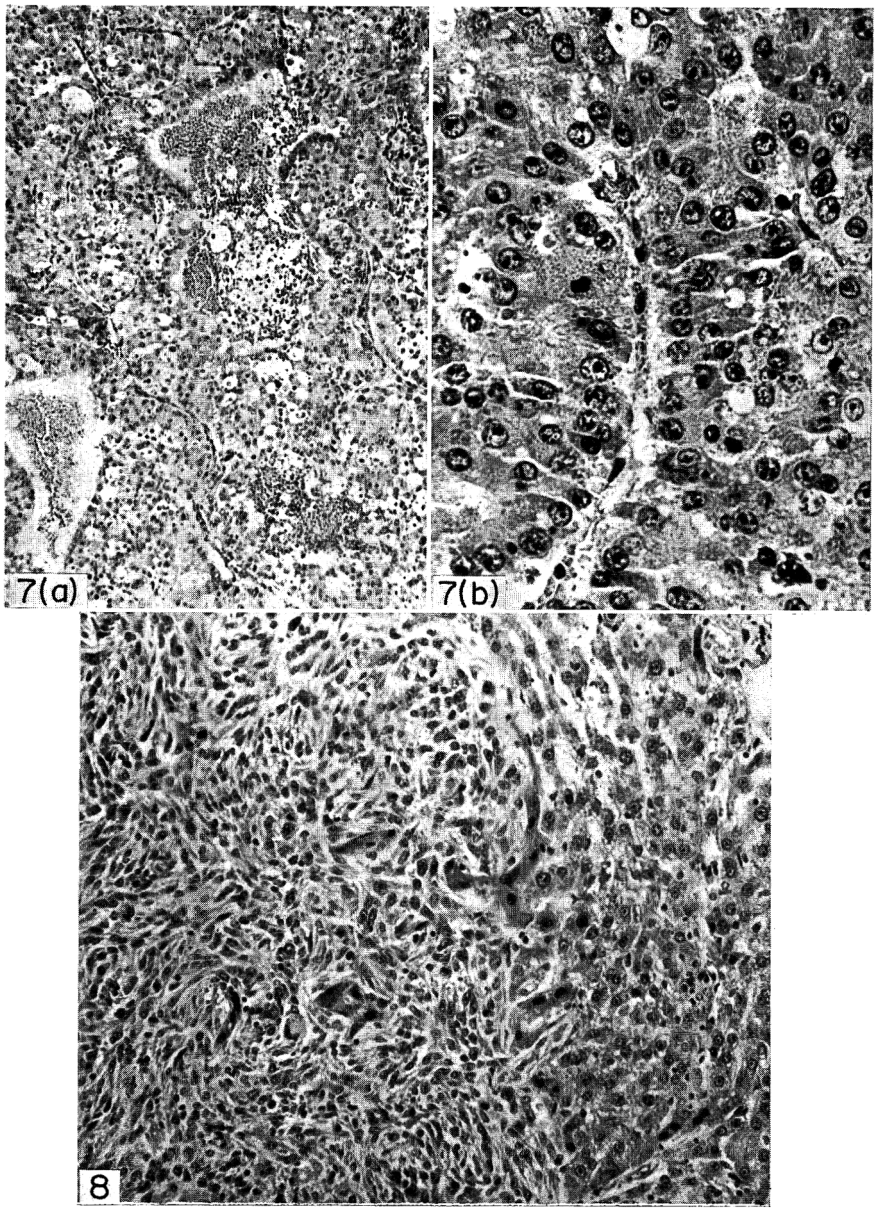


FIG. 7. Low-power photomicrograph (a) of a hepatocellular carcinoma from a rat given the copper-deficient–DMN treatment for 8 months, showing cystic spaces filled with cellular debris which were a feature of the malignant neoplasms. Higher magnification (b) illustrates cellular pleomorphism. Haematoxylin and eosin ×100 (a) and ×400 (b).

FIG. 8. Highly cellular fibrosarcoma primary in liver of a rat given the copper-deficient diet and DMN treatment for 6 months. Haematoxylin and eosin ×160.

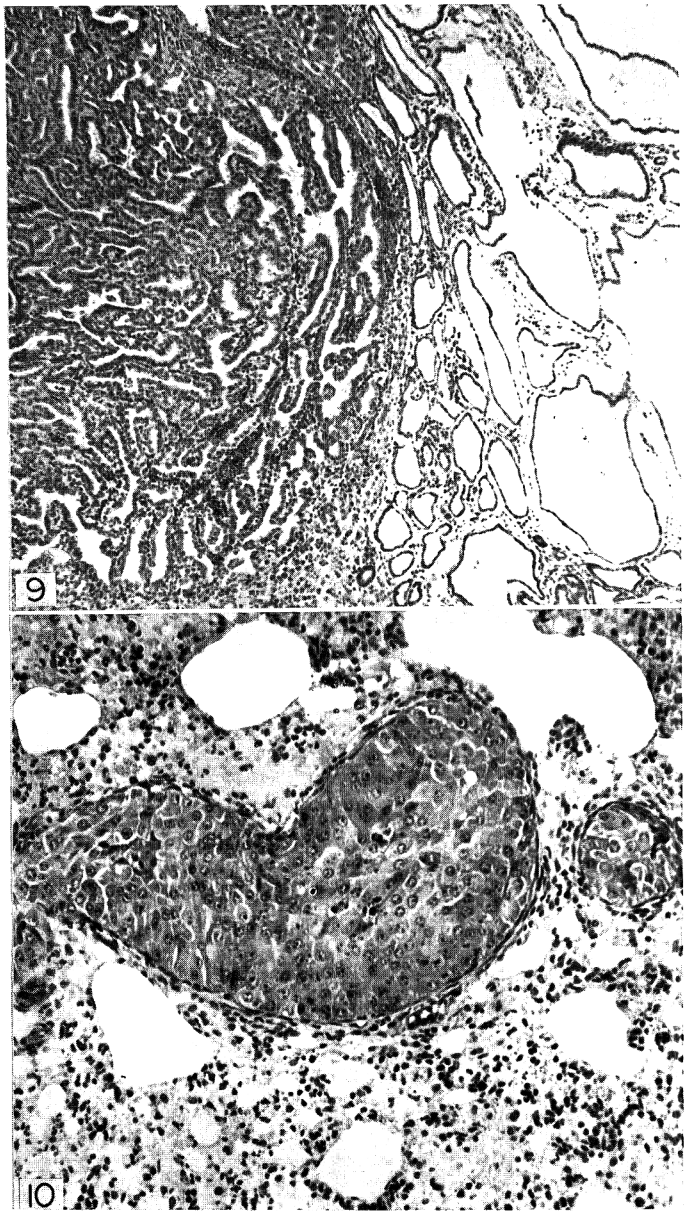


FIG. 9. Cholangioma consisting of small circumscribed masses of neoplastic biliary ducts from a rat fed the copper-deficient–AAF diet for 8 months. Many cysts were present in the livers of AAF-treated rats. Haematoxylin and eosin ×64.

FIG. 10. Multiple emboli of hepatocellular carcinoma in the veins of the lung from a rat fed the excess-copper–AAF diet for 8 months. Haematoxylin and eosin ×160.

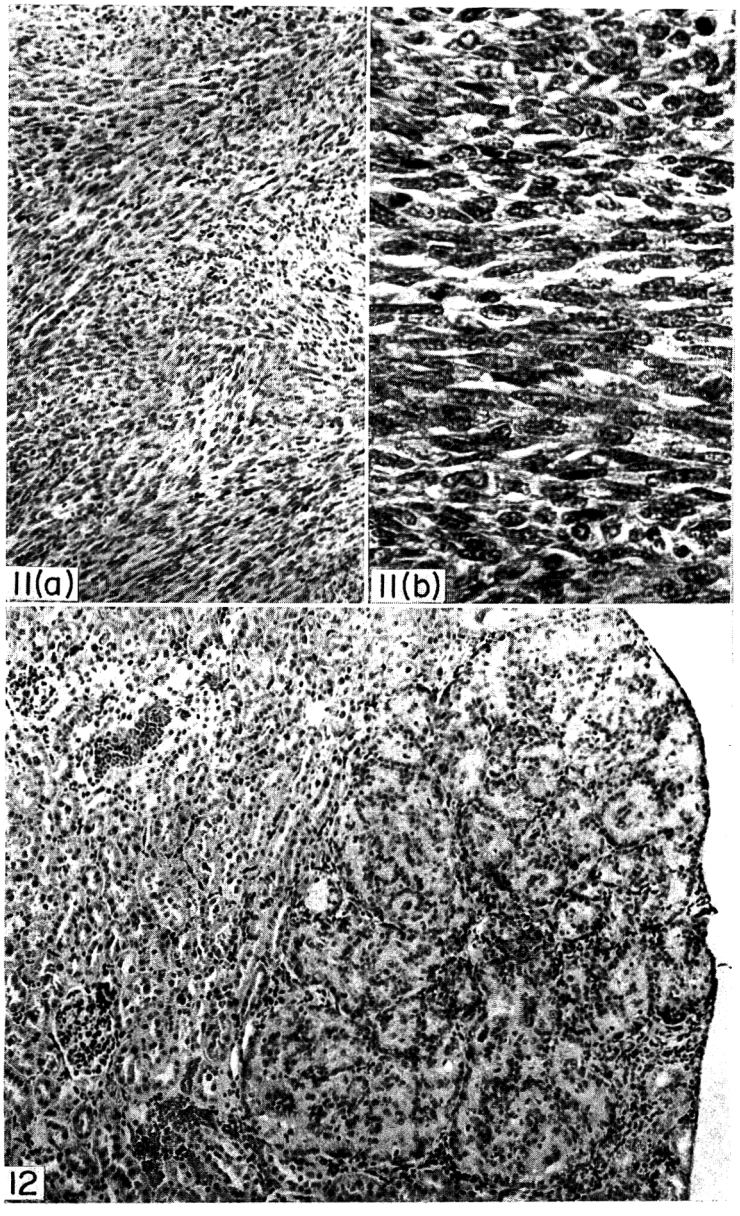
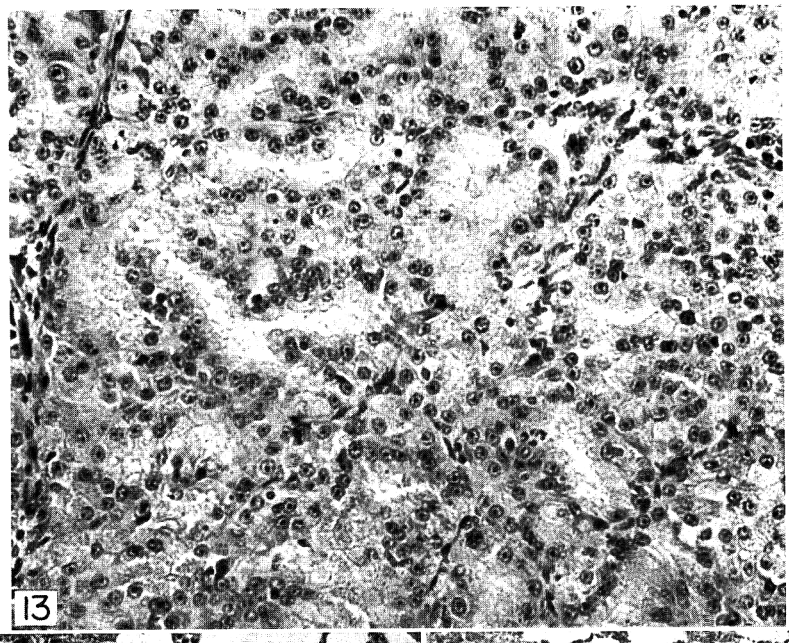
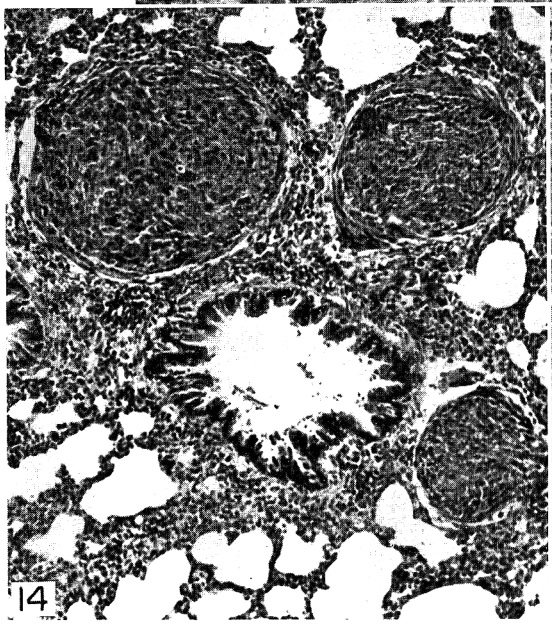


FIG. 11. A fibrosarcoma (the most common renal neoplasm) from a rat given the copper-deficient–DMN treatment and composed of interlacing bundles of cells (a), with the morphologic features of fibroblasts (b). Haematoxylin and eosin ×100 (a) and ×400 (b).

FIG. 12. Small nodules of proliferated tubular epithelium, considered to be neoplastic but non-malignant, from a rat given the copper-deficient–DMN treatment for 8 months. These adenomas were located at the capsular surface and within the renal cortex. Haematoxylin and eosin ×100.





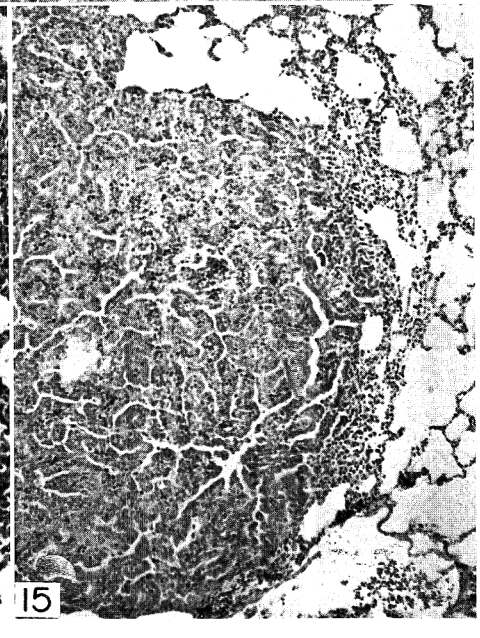


FIG. 13. Larger nodules displaying features of anaplasia and considered to be adenocarcinomas, from kidney of a rat given the copper-deficient–DMN treatment for 7 months. Haematoxylin and eosin ×264.

FIG. 14. Three pulmonary veins containing tumour emboli of a fibrosarcoma primary in the kidney, from a rat given the copper-deficient–DMN treatment for 7 months. Haematoxylin and eosin ×100.

FIG. 15. Adenoma arising from alveolar cells in a rat given the copper-deficient–DMN treatment for 6 months. Haematoxylin and eosin ×64.

Table 6. *Incidence of hepatic lesions and neoplasms in rats fed copper-deficient and excess-copper diets with DMN or AAF treatment and killed at monthly intervals for autopsy*

Experimental group	Total no. of rats killed	Incidence (%)* of						
		Liver necrosis	Transitional nodules	Hepatomas	Hepatocellular carcinomas	Metastases	Kidney neoplasms	Other neoplasms
Copper deficient diet:								
Control	42	0·0	0·0	0·0	0·0	0·0	2·4	0·0
+ DMN	30	30·8	76·7	23·3	10·0	0·0	56·7	30·0
+ AAF	27	22·2	100·0	92·6	40·7	3·7	0·0	40·0
Excess-copper diet:								
Control	32	9·4	3·1	0·0	0·0	0·0	0·0	0·0
+ DMN	29	55·2	82·8	27·6	13·8	0·0	0·0	24·1
+ AAF	30	30·0	100·0	90·0	30·0	10·0	0·0	16·7

DMN—50 ppm dimethylnitrosamine in drinking-water for 4-day periods alternating with distilled water.
AAF—0·06% acetylaminofluorene in the diet for 4-day periods alternating with control diet.

*Percentage of rats affected.

DMN was toxic at the level given, mean body weights being reduced and mortality increased compared with the copper-deficient control group. The combination of carcinogen and excess copper appeared not to be additive, although total mortality was slightly greater in the excess-copper group. The number of deaths was greater in the DMN-treated groups of this study than has been reported by others (Argus & Hoch-Ligeti, 1961; Ito, Johno, Marugami, Konishi & Hiasa, 1966; Zak, Holzner, Singer & Popper, 1960). The mode of administration, the age of the animals at the beginning of administration and the basal diet were variables that may have altered the response to the carcinogen. The mortality rate after 3 months was less in both the DMN groups and it appeared that rats surviving the early toxic effects developed some tolerance.

AAF produced a greater reduction in weight than DMN and the inhibition of weight gain was similar to that found by others (Bielschowsky, 1944; Miller, Miller & Hartmann, 1961; Wilson, DeEds & Cox, 1941). The administration of AAF and excess copper had an additive toxic effect as this diet markedly decreased body-weight gains, producing the lowest means in the experimental groups. These results differ from the findings of Fare & Howell (1964) and of Goodall (1964), but these investigators used different carcinogens.

Mortality over the experimental period did not vary greatly between the copper-deficient–AAF and the excess-copper–AAF groups. These data illustrate one of the differences in the response of rats to the two carcinogens. Excess copper in the diet markedly increased the mortality rate after DMN administration but appeared to have little effect when AAF was fed.

Enlargement of the liver was restricted, with few exceptions, to the AAF-fed rats and the mean liver weights were within the range of values reported by Webster, Lijegren & Zimmer (1947). DMN administration slightly increased the mean liver weight expressed as a percentage of body weight and our data on the liver weights of rats receiving DMN are similar to the findings of Ito *et al.* (1966). Livers from AAF-fed rats were generally enlarged, some greatly. The rats receiving AAF had the lowest body weights and little body fat. These factors, combined with the presence of many cysts and neoplasms, account for the high liver-weight values observed in the AAF-fed groups (Table 2).

The copper content of the livers from rats fed the copper-deficient diets did not vary greatly. However, the hepatic copper concentration of excess-copper control rats was less than the mean concentrations for the two carcinogen-treated groups. Thus, the carcinogens appeared to increase the retention of copper by the liver in animals receiving the excess-copper diet. The copper levels of non-neoplastic and grossly neoplastic hepatic tissue from rats fed the copper-deficient diet and treated with DMN or AAF were similar, but the copper content of non-neoplastic hepatic tissue from rats fed the excess-copper diet with AAF was greater than that of neoplastic tissue. Whether the copper content of chemically-induced neoplastic tissue is higher or lower than normal tissue appears to vary with the carcinogen, the normal tissue and the type of neoplasm induced (Arnold & Sasse, 1961; Fare & Woodhouse, 1953; Greenstein & Thompson, 1943; Pedrero & Kozelka, 1951). The copper content of the renal tissues decreased in the following order: normal tissue in copper-deficient control rats, non-neoplastic tissue in copper-deficient–DMN rats, DMN-induced small neoplasms and DMN-induced large neoplasms (over 22 g). Part of the lower copper content may be due to the tissue composition of the neoplasms; fibrosarcomas are composed of connective tissue known to have a low copper content.

The incidence of hepatic neoplasms in DMN-treated rats was similar for the copper-deficient and excess-copper groups. This contrasts with the report of Howell (1958) who

observed that a high-copper diet inhibited hepatic carcinogenesis by DMAB. The variables of a different carcinogen, older rats and a different basal diet, as well as a higher copper level, could partially account for the difference.

Histological classification of DMN-induced renal neoplasms by various researchers include two major types, although differences in morphology and incidence rates have been reported (Magee & Barnes, 1962). In the current study, neoplasms were found to originate from the epithelium of the renal tubules and the connective tissue of the interstitium. Those originating in the interstitial cells were classified as fibrosarcomas on the basis of morphology and staining reactions. The generic name of "stromal nephroma" has been proposed for tumours that arise from the interstitial cells (Riopelle & Jasmin, 1969). Hard & Butler (1971) reported the development of DMN-induced renal mesenchymal tumours from interstitial cells with fibroblast morphology. Seventeen rats killed for autopsy had one or more neoplasms, including 12 fibrosarcomas, seven adenomas and two adenocarcinomas. These findings differ from the results of Argus & Hoch-Ligeti (1961) who observed 11 renal adenomas and only one sarcoma. Adenomas and sarcomas were of equal incidence in the study of Yang (1966).

AAF is a potent carcinogen, inducing neoplasms in several sites in the rat and other animal species (Bielschowsky, 1944 & 1947; Engel & Copeland, 1951; Miller *et al.* 1961; Weisburger & Weisburger, 1963; Wilson *et al.* 1941). These observations were confirmed by the present research, and organs with neoplasms included the liver, spleen, lung, skin, muscle, pancreas and intestine, although neoplasms were uncommon in the last three organs. The numbers of hepatic neoplasms in AAF-treated rats on the copper-deficient and excess-copper diets were similar and it appeared that the concentration of copper had no effect upon the incidence of hepatic neoplasms. However, the latency period may have been slightly increased, as hepatocellular carcinomas and metastases occurred 1 month later in the excess-copper group.

The incidence of extrahepatic neoplasms in rats killed for autopsy was 40% in the copper-deficient–AAF group, but only 17% of the rats fed the excess-copper–AAF diet had neoplasms outside the liver. When the extrahepatic tumours from rats found dead after receiving the AAF treatment for at least 3 months are combined with those from rats killed for autopsy, the difference in incidence of neoplasms between copper-deficient and excess-copper groups was decreased (31 *v.* 23%) but the data suggest that the copper supplement acted to reduce the number of extrahepatic neoplasms.

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REFERENCES

Argus, Mary F. & Hoch-Ligeti, Cornelia (1961). Comparative study of the carcinogenic activity of nitrosamines. *J. natn. Cancer Inst.* **27**, 695.

Arnold, M. & Sasse, D. (1961). Quantitative and histochemical analysis of Cu, Zn, and Fe in spontaneous and induced primary tumors of rats. *Cancer Res.* **21**, 761.

Bielschowsky, F. (1944). Distant tumors produced by 2-amino- and 2-acetylaminofluorene. *Br. J. exp. Path.* **25**, 1.

Bielschowsky, F. (1947). The carcinogenic action of 2-acetylaminofluorene and related compounds. *Br. med. Bull.* **4**, 382.

Boyden, R., Potter, V. R. & Elvehjem, C. A. (1938). Effect of feeding high levels of copper to albino rats. *J. Nutr.* **15**, 397.

Engel, R. W. & Copeland, D. H. (1951). Influence of diet on the relative incidence of eye, mammary, ear-duct, and liver tumors in rats fed 2-acetylaminofluorene. *Cancer Res.* **11,** 130.
Fare, G. & Howell, J. S. (1964). The effect of dietary copper on rat carcinogenesis by 3-methoxy dyes. I. Tumors induced at various sites by feeding 3-methoxy-4-aminoazobenzene and its *N*-methyl derivative. *Cancer Res.* **24,** 1279.
Fare, G. & Woodhouse, D. L. (1963). The effect of copper acetate on biochemical changes induced in the rat liver by *p*-dimethylaminoazobenzene. *Br. J. Cancer* **17,** 512.
Goodall, C. M. (1964). Failure of copper to inhibit carcinogenesis by 2-aminofluorene. *Br. J. Cancer* **18,** 777.
Greenblatt, M., Mirvish, S. & So, B. T. (1971). Nitrosamine studies: Induction of lung adenomas by concurrent administration of sodium nitrite and secondary amines in Swiss mice. *J. natn. Cancer Inst.* **46,** 1029.
Greenstein, J. P. & Thompson, J. W. (1943). Note on the copper content of the tissues of tumor-bearing animals. *J. natn. Cancer Inst.* **3,** 405.
Hard, G. C. & Butler, W. H. (1971). Ultrastructural study of the development of interstitial lesions leading to mesenchymal neoplasia induced in the rat renal cortex by dimethylnitrosamine. *Cancer Res.* **31,** 337.
Howell, J. S. (1958). The effect of copper acetate on *p*-dimethylaminoazobenzene carcinogenesis in the rat. *Br. J. Cancer* **12,** 594.
Ito, N., Johno, I., Marugami, M., Konishi, Y. & Hiasa, Y. (1966). Histopathological and autoradiographic studies on kidney tumors induced by *N*-nitrosodimethylamine in rat. *Gann* **57,** 595.
King, H. J., Spain, J. D. & Clayton, C. D. (1957). Dietary copper salts and azo dye carcinogenesis. *J. Nutr.* **63,** 301.
Klubes, P. & Jondorf, W. R. (1971). Dimethylnitrosamine formation from sodium nitrite and dimethylamine by bacterial flora of rat intestine. *Res. Commun. chem. Path. Pharmac.* **2,** 24.
Magee, P. N. & Barnes, J. M. (1962). Induction of kidney tumours in the rat with dimethylnitrosamine (*N*-nitrosodimethylamine). *J. Path. Bact.* **84,** 19.
Miller, Elizabeth C., Miller, J. A. & Hartmann, H. A. (1961). *N*-Hydroxy-2-acetylaminofluorene: A metabolite of 2-acetylaminofluorene with increased carcinogenic activity in the rat. *Cancer Res.* **21,** 815.
Mills, C. F. & Murray, Grace (1960). The preparation of a semi-synthetic diet low in copper for copper-deficiency studies with the rat. *J. Sci. Fd Agric.* **11,** 547.
Parker, M. M., Humoller, F. L. & Mahler, D. J. (1967). Determination of copper and zinc in biological material. *Clin. Chem.* **13,** 40.
Pedrero, E., Jr. & Kozelka, F. L. (1951). Effect of copper or hepatic tumors produced by 3'-methyl-4-dimethylaminoazobenzene. *Archs Path.* **52,** 455.
Riopelle, J. L. & Jasmin, G. (1969). Nature, classification, and nomenclature of kidney tumors induced in the rat by dimethylnitrosamine. *J. natn. Cancer Inst.* **42,** 643.
Ross, M. H. & Bras, G. (1965). Tumor incidence patterns and nutrition in the rat. *J. Nutr.* **87,** 245.
Sander, J. u. Bürkle, G. (1969). Induktion maligner Tumoren bei Ratten durch gleichzeitige Verfütterung von Nitrit und sekundären Aminen. *Z. Krebsforsch.* **73,** 54.
Webster, S. H., Liljegren, E. J. & Zimmer, D. J. (1947). Organ; body weight ratios for livers, kidneys, and spleens of laboratory animals. *Am. J. Anat.* **81,** 477.
Weisburger, J. H. & Weisburger, Elizabeth K. (1963). Pharmacodynamics of carcinogenic azo dyes, aromatic amines, and nitrosamines. *Clin. Pharmac. Ther.* **4,** 110.
Wilson, R. H., DeEds, F. & Cox, A. J., Jr. (1941). The toxicity and carcinogenic activity of 2-acetaminofluorene. *Cancer Res.* **1,** 595.
Wolff, S. M. (1960). Copper deposition in the rat. *Archs Path.* **69,** 217.
Yang, Y. H. (1966). Renal hyperplasia and neoplasia in rats given dimethylnitrosamine. *Urol. Int.* **21,** 229.
Zak, F. G., Holzner, J. H., Singer, E. J. & Popper, H. (1960). Renal and pulmonary tumors in rats fed dimethylnitrosamine. *Cancer Res.* **20,** 96.

Le cuivre alimentaire et l'induction de néoplasmes chez le rat par l'acétylaminofluorène et la diméthylnitrosamine

Résumé—On a étudié l'effet du cuivre alimentaire sur la fréquence et la localisation des néoplasmes chez des rats soumis à un régime déficient en cuivre (1 ppm) et à un régime à excès de cuivre (800 ppm) contenant de l'acétylaminofluorène (AAF), ainsi que chez des rats soumis aux mêmes régimes, pauvre et trop riche en cuivre, mais dont l'eau de boisson avait été additionnée de diméthylnitrosamine (DMN) pendant 9 mois. Avec ou sans DMN ou AAF, le cuivre en excès s'est révélé toxique en réduisant le gain de poids et en faisant augmenter la mortalité. Le ralentissement du gain de poids dû à l'excès de cuivre a été le plus marqué dans le groupe AAF, mais la mortalité la plus élevée (72%) s'est manifestée dans le groupe DMN.

Le poids du foie a augmenté chez les rats traités à l'AAF, en partie à cause de la présence de néoplasmes. Le teneur moyenne en cuivre du tissu hépatique des rats soumis aux régimes déficients en cuivre était plus élevée (4,5 ppm) que chez les animaux traités à la DMN (3,9 ppm) ou à l'AAF (2,8 ppm). La concentration du cuivre dans le foie des rats soumis aux régimes à excès de cuivre s'est élevée en moyenne à 244 ppm chez les animaux témoins et les taux correspondants étaient de 394 ppm pour le groupe traité à la DMN et de 354 ppm pour le groupe traité à l'AAF. La teneur en cuivre du tissu hépatique néoplasique des rats soumis au régime déficient en cuivre était similaire à celle du tissu non néoplasique, mais chez les rats soumis au régime à excès de cuivre le tissu hépatique néoplasique contenait moins de celui-ci que le foie non néoplasique. Le tissu néoplasique des reins contenait moins de cuivre que le tissu rénal normal, dans l'ensemble. Les fréquences de néoplasmes hépatiques étaient similaires chez les rats soumis aux deux régimes, pauvre et trop riche en cuivre, et traités à la DMN, mais des néoplasmes des reins ont été constatés chez 57% des rats du groupe à déficience de cuivre traités à la DMN et sacrifiés aux fins d'autopsie; cette fréquence était de 0,0% dans le groupe à excès de cuivre traité à la DMN. Cette différence de fréquence pourrait être en corrélation avec les taux de cuivre des régimes. Les fréquences de néoplasmes pulmonaires provoqués par la DMN étaient similaires chez les rats du groupe à régime pauvre en cuivre et chez ceux du groupe à régime à excés de cuivre. Des néoplasmes hépatiques ont été constatés chez plus d'animaux traités à l'AAF que d'animaux traités à la DMN, mais la teneur en cuivre du régime n'a eu aucun effet sur la fréquence. Les néoplasmes non hépatiques constatés chez les rats traités à l'AAF se situaient dans les poumons, la rate, la peau, l'intestin, le pancréas et les muscles. La fréquence des néoplasmes non hépatiques était de 40% chez les rats soumis au régime déficient en cuivre et traités à l'AAF sacrifiés aux fins d'autopsie, mais ne s'est élevée qu'à 17% chez les rats soumis au régime à excès de cuivre.

Kupfergehalt des Futters und Induktion von Neoplasmen bei der Ratte durch Acetylaminofluoren und Dimethylnitrosamin

Zusammenfassung—Die Wirkung des Kupfergehalts des Futters auf die Häufigkeit und Lokalisierung von Neoplasmen wurde an Ratten untersucht, die kupferarmes (1 ppm) und einen Kupferüberschuss (800 ppm) enthaltendes Futter mit Acetylaminofluoren (AAF) und an Ratten, die das gleiche kupferarme und einen Kupferüberschuss enthaltende Futter und dazu Trinkwasser mit Dimethylnitrosamin (DMN) 9 Monate lang erhielten. Der Kupferüberschuss mit oder ohne DMN oder AAF war toxisch, die Körpergewichtszunahme vermindert und die Mortalität erhöht. Die Verminderung der Körpergewichtszunahme war am grössten in der Kupferüberschuss–AAF-Gruppe, jedoch trat die höchste Mortalität (72%) in der Kupferüberschuss–DMN-Gruppe auf. Das Lebergewicht war bei den Ratten, die AAf erhielten, erhöht, teilweise wegen des Vorhandenseins von Neoplasmen. Der durchschnittliche Kupfergehalt (4,5 ppm) in Lebergewebe von Ratten, die das kupferarme Futter erhalten hatten, war grösser als der im Lebergewebe von DMN-behandelten (3,9 ppm) oder mit AAF gefütterten (2,8 ppm) Ratten. Die Kupferkonzentration in der Leber von Ratten, die das Futter mit Kupferüberschuss erhalten hatten, betrug durchschnittlich bei den Kontrolltieren 244 ppm, während die vergleichbaren Zahlen für die DMN-behandelten und mit AAF gefütterten Ratten 394 bzw.354 ppm betrugen. Neoplastisches Lebergewebe von Ratten, welche das kupferarme Futter erhalten hatten, besass einen ähnlichen Kupfergehalt wie das nichtneoplastische Gewebe, aber das neoplastische Lebergewebe von Ratten mit der Kupferüberschussfütterung war niedriger im Kupfergehalt als das von nichtneoplastischer Leber. Neoplastisches Gewebe von der Niere wies weniger Kupfer auf als makroskopisch normales Nierengewebe. Die Häufigkeit von Leberneoplasmen war bei DMN-behandelten Ratten, die entweder das kupferarme oder das kupferüberschüssige Futter erhalten hatten, vergleichbar, aber Nierenneoplasmen traten bei 57% der Ratten auf, welche kupferarmes Futter und DMN erhalten hatten und zwecks Autopsie getötet wurden, verglichen mit einer Häufigkeit von 0,0% in der Kupferüberschuss–DMN-Gruppe. Dieser Häufigkeitsunterschied kann mit den Kupferkonzentrationen im Futter in Verbindung gebracht werden. Die Häufigkeit von DMN-induzierten Neoplasmen in der Lunge bei Ratten, die kupferarmes, und solchen, die kupferüberschüssiges Futter erhalten hatten, war vergleichbar. Mehr AAF-gefütterte als DMN-behandelte Tiere entwickelten Neoplasmen der Leber, aber die Kupferkonzentration im

Futter hatte keinen Einfluss auf die Häufigkeit. Ausserhalb der Leber traten bei Ratten, die AAF im Futter erhalten hatten, Neoplasmen in der Lunge, in der Milz, in der Haut, im Darm, in der Bauchspeicheldrüse und in den Muskeln auf. Die Häufigkeit von Neoplasmen ausserhalb der Leber betrug 40% bei den zwecks Autopsie getöteten Ratten, die kupferarmes Futter mit AAF, aber nur 17% bei denen, die Futter mit Kupferüberschuss erhalten hatten.

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SHORT PAPERS

Dimethylnitrosamine in Chinese Marine Salt Fish

Y. Y. FONG and W. C. CHAN

Departments of Biochemistry and Pathology, University of Hong Kong, Hong Kong

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Summary—Samples of marine salt fish from the markets in Hong Kong were analysed for *N,N*-dimethylnitrosamine (DMNA). Significant amounts of this compound, commonly in the 0·05–0·3 ppm range, were detected by gas chromatography and were confirmed in several samples by gas chromatography–mass spectrometry. Salt fish prepared with crude salt contained much more DMNA than that prepared with pure sodium chloride.

Introduction

Nitrosamines have been identified and confirmed in various amounts in many types of prepared food (Fazio, Damico, Howard, White & Watts, 1971; Sen, 1972; Wasserman, Fiddler, Doerr, Osman & Dooley, 1972). Some methods of preparation are conducive to the formation of nitrosamines. The preparation of Cantonese marine salt fish, a favourite dish along the South China coast and also in South-east Asian countries is a good example. In the fish itself there are rich sources of secondary and tertiary amines (Rea & Shewan, 1949; Varela & Wojciech, 1956), while in the crude salt used to pickle the fish, there is nitrate and possibly nitrite. The pickling and drying is done in the open, so that the fish is liable to contamination by bacteria. All these factors suggest the possibility that nitrosamines might be formed in the salt fish.

The present paper reports the detection and confirmation of dimethylnitrosamine (DMNA) in marine salt fish obtained from local markets and an attempt to study the possible mechanism of its formation.

Experimental

Materials. Different species of Cantonese salt fish were purchased from local retailers The fish (obtained whole) were chopped into small pieces and homogenized with 20% potassium carbonate solution using a Sorvall Omni-Homogenizer. For each experiment, unless otherwise specified, a total of 4 kg fish was used. Nitrosamines extraction was carried out according to Sen, Smith, Schwinghamer & Howsam (1970). Briefly, the sample was first treated with methylene chloride, then warmed to remove the solvent and steam-distilled, the distillate was passed through a combined ion-exchange polyamide column and the combined effluent was extracted five times with methylene chloride, dried over anhydrous sodium sulphate and concentrated.

Analytical procedures. Quantitative measurements for DMNA were carried out with a Varian Aerograph gas chromatograph, Model 1864-4, using two different columns: (i) a 6 ft × 1/8 in. stainless-steel column packed with 5% SE30 on high-performance grade Chromosorb W/AW-DMCS (80–100 mesh), using a nitrogen flow of 28 ml/min and

column, injection and detector temperatures of 48, 80 and 125°C, respectively (range 10, attenuation 1); (ii) a 10 ft × 1/8 in. stainless-steel column packed with 10% Carbowax 20 M on high-performance grade Chromosorb W/AW-DMCS (80–100 mesh), with a nitrogen flow of 25 ml/min, and column, injection and detector temperatures of 95, 140 and 170°C, respectively (range 10, attenuation 1). The presence of DMNA was confirmed by the use of the same gas chromatograph with a 10% Carbowax 20 M stainless-steel column interfaced with a Hitachi Perkin–Elmer RMS-4 mass spectrometer. Helium carrier gas was used at a flow rate of 25 ml/min, and hydrogen- and air-flow conditions were 40 and 350 ml/min, respectively. The column effluent was split approximately 1: 9, 90% passing into the mass spectrometer via an inlet line heated at 200°C. The mass spectrum was obtained at an ionizing voltage of 70 eV.

Nitrate and nitrite determinations. Nitrate and nitrite determinations were carried out on six different batches of salt commonly used to prepare salt fish, on the salt scraped from the salt fish and on the salt fish themselves. The method adopted was that of Follett & Ratcliff (1963).

Isolation and identification of bacterial flora of salt fish. Inner portions of salt fish, removed with a pair of sterile forceps, were emulsified in sterile water by grinding with a sterile pestle and mortar and were then cultured in a 10% NaCl infusion broth at 37°C for 1 day. Subcultures were then prepared on various 10–25% salt milk-agar plates and incubated at 37°C. The usual morphological, staining, culture and biochemical tests were performed on the bacteria.

Salting of fish with laboratory NaCl and market-bought crude salt. Two different species of marine fish, white herring and yellow croaker (both dead), were purchased from local retailers. Equal portions of each were salted with crude salt and with chemically pure laboratory salt, respectively. The usual method of preparation practised by local fishermen was followed (McCarthy & Tausz, 1952). The fish were not gutted, nor were the gills removed. The fish were pickled for 1–2 days by putting layers of salt between them. They were then dried in the open air under the sun for a week or so depending on the weather. Just prior to drying, solid salt was forced into the mouth and between the gills. The fish were then put away and stored in the kitchen for 4–5 months, before being analysed for the presence of DMNA by the method already described.

Results

Table 1 shows the concentration of nitrite, nitrate and DMNA in the different batches of salt fish purchased from the local retailers. In white herring, batch G, the level of DMNA reached 1 ppm, but this could not serve as a typical example since batch G was only half a fish (head, weight 80 g), chosen for analysis because of its highly spoiled condition. The identification of DMNA from several batches was based on thin-layer and gas–liquid chromatography only, as the combined gas chromatography–mass spectrometry (GC–MS) technique was not available at that stage of our study. However, the batches marked with daggers were confirmed by the GC–MS technique; the m/e peaks corresponding to M^+ (74) and NO^+ (30) were distinctly visible over the background signals.

Levels of nitrate were found to be appreciable in the crude salt commonly used to prepare salt fish, amounting to 17, 18, 20, 20, 30 and 40 ppm in six samples of such salt purchased from local retailers. The nitrite content of these samples was insignificant, however, reaching no more than 1 ppm.

Table 1. *Dimethylnitrosamine concentrations in salt fish obtained from local markets*

Fish samples	DMNA* (ppm)	Residual nitrite (ppm)	Residual nitrate (ppm)
White herring			
A	0·30	1	6
B	0·20	1	10
C	0·05†	2	30
D	0·10†	1	30
E	0·04†	—	40
F	0·06†	—	8
G	1·00	2	10
Yellow croaker			
A	0·20	1	30
B	0·01†	1	18
C	0·05†	1	20
D	0·06†	4	15
E	0·08	2	30
Anchovies			
A	0·10	2	10
B	0·02†	1	8
Croaker			
A	0·03	2	10
B	0·02†	—	20
Pomfret			
A	Not detected	1	30

*N,N-Dimethylnitrosamine, corrected for recovery of 70% determined experimentally.
†Identity confirmed by gas chromatography–mass spectrometry.

The difference between the DMNA level in salt fish prepared with chemically pure salt and that in fish prepared with crude salt is shown in Table 2. The identity of the DMNA was established by GC–MS.

Table 2. *Differences in DMNA levels in salt fish prepared with crude salt and NaCl*

Fish	No. of samples analysed	DMNA* (ppm) in fish prepared with	
		Crude salt	NaCl
White herring	1	0·4†	0·01†
Yellow croaker	1	0·2†	0·005†

*N,N-Dimethylnitrosamine, corrected for recovery of 70% determined experimentally.
†Confirmed by gas chromatography–mass spectrometry.

The nitrate-reducing bacteria isolated from the fish were identified as a coagulase-positive *Staphylococcus aureus*.

Discussion

The presence of DMNA demonstrated in Cantonese marine salt fish obtained from markets in Hong Kong was to be anticipated from the conditions under which the fish is prepared. The level of DMNA varied greatly and, with one exception, all the batches of fish examined showed significantly higher DMNA levels than those reported for preserved fish elsewhere (Crosby, Foreman, Palframan & Sawyer, 1972; Fazio *et al.* 1971). The variation in DMNA content was related in part to the degree of contamination by nitrate-reducing staphylococci. This organism isolated from salt fish has been shown to increase the DMNA content in salt-fish broth (Fong & Chan, 1973). In the present survey, the most spoiled batch contained the largest amount of DMNA.

The nitrate present as an impurity in the pickling salt is a precursor of DMNA. This was demonstrated in the experiment in which both crude salt and pure NaCl were used to pickle fish. Salt fish prepared by using crude salt, which contains 17–40 ppm nitrate but not more than 1 ppm nitrite was shown to contain 40 times more DMNA than the same fish pickled.

Different species of fish showed different levels of DMNA. Salted white herring contained more DMNA than salted yellow croaker, while a single batch of pomfret contained no detectable DMNA at all. It is possible that the contents of secondary and tertiary amines, both precursors of nitrosamine, may vary widely in different species of fish (Rea & Shewan, 1949; Shewan, 1937).

The above observations suggest that the prerequisites for DMNA production in salt fish may be the availability of secondary amines in the fish, the presence of nitrate in the crude salt used for pickling and the intervention of nitrate-reducing staphylococci or other bacteria. *In vitro* formation of nitrosamines from its precursors by bacteria has been demonstrated by several investigators (Collins-Thompson, Sen, Aris & Schwinghamer, 1972; Hawksworth & Hill, 1972; Sander, 1968).

The source of staphylococci is probably contamination during preparation of the fish. McCarthy & Tausz (1952) studied the salt-fish industry in Hong Kong and commented on the unhygienic way the fish are dried in the open, under the sun. Another source may be the gut of the fish, which are not gutted. The same authors suggested for hygienic reasons that the drying should be done in mechanical driers. We have now a stronger reason to recommend this, as the reduction of infection would lead to a reduction in nitrosamine levels.

Hong Kong is an area where the incidence of liver cell carcinoma and nasopharyngeal carcinoma is high. Whether the DMNA in salt fish may play a causative role in this carcinogenesis remains to be proven. The amount of salt fish consumed at any one time is small—not more than 10 g—but in some families this dish may appear at every meal and some people may develop a taste for it and actually prefer the spoiled part. In these circumstances, the intake of DMNA over the years may be significant.

Acknowledgements—We wish to express our appreciation to the Chemistry Department, University of Hong Kong, for the use of their equipment, to Dr. R. S. M. Tse for advice on gas chromatography–mass spectrometry and to Mr. S. C. Wong for technical assistance with the mass spectrometer.

REFERENCES

Collins-Thompson, D. L., Sen, N. P., Aris, B. & Schwinghamer, L. (1972). Non-enzymic in vitro formation of nitrosamines by bacteria isolated from meat products. *Can. J. Microbiol.* **18,** 1968.

Crosby, N. T., Foreman, J. K., Palframan, J. F. & Sawyer, R. (1972). Estimation of steam-volatile N-nitrosamines in foods at the 1 μg/kg level. *Nature, Lond.* **238,** 342.

Fazio, T., Damico, J. N., Howard, J. W., White, R. H. & Watts, J. O. (1971). Gas chromatographic determination and mass spectrometric confirmation of N-nitrosodimethylamine in smoke-processed marine fish. *J. agric. Fd Chem.* **19,** 250.

Follett, M. J. & Ratcliff, P. W. (1963). Determination of nitrite and nitrate in meat products. *J. Sci. Fd Agric.* **14,** 138.

Fong, Y. Y. & Chan, W. C. (1973). Bacterial production of di-methyl nitrosamine in salted fish. *Nature, Lond.* **243,** 421.

Hawksworth, Gabrielle M. & Hill, M. J. (1972). Bacteria and the N-nitrosation of secondary amines. *Br. J. Cancer* **25,** 520.

McCarthy, J. P. & Tausz, J. (1952). Salt Fish Industry in Hong Kong. The Government Printer, Hong Kong.

Rea, G. A. & Shewan, J. M. (1949). The spoilage of fish and its preservation by chilling. *Adv. Fd Res.* **2,** 343.

Sander, J. (1968). Nitrosaminsynthese durch Bakterien. *Hoppe-Seyler's Z. physiol. Chem.* **349,** 429.

Sen, N. P. (1972). The evidence for the presence of dimethylnitrosamine in meat products. *Fd Cosmet. Toxicol.* **10,** 219.

Sen, N. P., Smith, Dorothy C., Schwinghamer, L. & Howsam, Barbara (1970). Formation of nitrosamines in nitrite-treated fish. *Can. Inst. Fd Technol. J.* **3,** 66.

Shewan, J. M. (1937). The spoilage of haddocks stowed in ice. *Rep. Fd Invest. Bd,* p. 75.

Varela, G. & Wojciech, R. (1956). Biochemical process in the decomposition of European hake (Merluccius merluccius). *An. Bromat.* **8,** 5.

Wasserman, A. E., Fiddler, W., Doerr, R. C., Osman, S. F. & Dooley, C. J. (1972). Dimethylnitrosamine in frankfurters. *Fd Cosmet. Toxicol.* **10,** 681.

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Hyperplasia of Hepatic Bile Ducts in Mice Following Long-term Administration of Butylated Hydroxytoluene*

N. K. CLAPP, R. L. TYNDALL and R. B. CUMMING

Biology Division, Oak Ridge National Laboratory, Oak Ridge, Tennessee 37830, USA

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Summary—Six of 18 male BALB/c mice regularly given a diet containing 0·75% butylated hydroxytoluene (BHT) and killed at 12 months of age showed a marked hyperplasia of the hepatic bile ducts, with an associated subacute cholangitis. None of the 64 untreated control mice nor 19 BHT-treated control mice that additionally received diethylnitrosamine in their drinking-water, developed proliferation of the bile-duct epithelium. While the cause, pathogenesis and fate of the observed lesion is uncertain, further investigation is needed to determine the possible hazard arising from long-term human consumption of BHT.

Introduction

Antioxidants are commonly added to human foods as preservatives for unsaturated lipids and other materials subject to spoilage by oxidation. One of these compounds is butylated hydroxytoluene (BHT), a widely used fat preservative which is included in the FDA's GRAS (generally recognized as safe) list. Some of these agents produce a variety of changes when fed to experimental animals at doses considerably higher than the doses used for food preservation. With BHT, these changes include reduced growth rate and body weight (Brown, Johnson & O'Halloran, 1959; Deichmann, Clemmer, Rakoczy & Bianchine, 1955; Frawley, Kohn, Kay & Calandra, 1965; Johnson & Hewgill, 1961), elevated serum cholesterol (Day, Johnson, O'Halloran & Schwartz, 1959; Frawley *et al.* 1965; Gaunt, Gilbert & Martin, 1965; Johnson & Hewgill, 1961), hypertrophy of the liver with increases in smooth endoplasmic reticulum and in the mitotic activity of hepatocytes (Botham, Conning, Hayes, Litchfield & McElligott, 1970; Brown *et al.* 1959; Deichmann *et al.* 1955; Feuer, Gaunt, Golberg & Fairweather, 1965; Gaunt *et al.* 1965; Gilbert & Golberg, 1965; Kerr, Lefevre, Lane & Lieber, 1966; Lane & Lieber, 1967) and alterations in the activity of several liver enzymes (Creaven, Davies & Williams, 1966; Feuer *et al.* 1965; Gilbert & Golberg, 1967; Gilbert, Martin, Gangolli, Abraham & Golberg, 1969; Nievel, 1969). The liver changes reported for BHT have been interpreted as nondeleterious (Deichmann *et al.* 1955), and there are no reports of late somatic effects. However, BHT has recently been reported to modify effectively the acute toxicity of a number of mutagenic and carcinogenic chemicals in the mouse (Cumming & Walton, 1973). The possibility still remains that the regular consumption of this or similar compounds for long periods may pose a health hazard to man.

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Experimental

As part of a larger study to determine ways to modify the carcinogenic activity of known chemical carcinogens, we treated a group of 18 8-wk-old male BALB/c mice continuously with 0·75% BHT in pelleted Purina Laboratory Chow (Ralston Purina, St. Louis, Mo.). A control group of 64 mice received the same feed without BHT and a further group of 19 mice was treated with BHT in the food and simultaneously given diethylnitrosamine (DENA) in the drinking-water in a total dose of 490 mg/kg body weight over a 7-wk period. The mice were killed at 12 months of age and complete autopsies were performed, including routine histological examination. The complete pathological findings will be reported elsewhere. Before the start of the experiment, sera from the mice were tested for the presence of antibodies to common murine viruses (Microbiological Associates, Bethesda, Md) with negative results; the mice were also found grossly and histologically to be free from ecto- and endoparasites.

Results and Discussion

Of 18 BHT-treated mice, six developed a marked hyperplasia of the hepatic bile ducts with an associated subacute cholangitis (Fig. 1). Finger-like projections of proliferating bile-duct epithelium extended through numerous areas of the liver. A bile duct of normal size with cuboidal epithelium is shown in higher magnification in Fig. 2, the cell size and shape contrasting with the hyperplastic epithelial cells, which are columnar with basal alignment of hypochromatic nuclei. The proliferative change existed throughout the liver and involved hepatic ducts only. The subacute inflammatory process was composed of lymphocytes, neutrophils and plasma cells intermingled with a mild fibroblastic activity and early collagen formation. Hepatocytes were vacuolated, suggesting that some lipid-like material had been removed during histological processing. None of the 64 untreated control animals showed this liver lesion, nor has it been seen in several thousand BALB/c and RF mice used in studies on other chemicals (Clapp, Craig & Toya, 1968 & 1970; Clapp & Toya, 1970; Clapp, Tyndall & Otten, 1971) and radiation (Clapp, 1973; Clapp, Darden & Jernigan, 1973). The mice treated simultaneously with BHT in the food and DENA in the drinking-water also failed to develop this lesion, suggesting that DENA may inhibit this BHT effect.

The aetiology, pathogenesis and ultimate fate of this bile-duct lesion are unknown at this time, but further studies are in progress to determine whether either BHT itself or a metabolite may be the causal agent. The frequency and severity of bile-duct hyperplasia in BHT-treated mice strongly suggests the need for further investigation of BHT as a possibly deleterious agent.

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REFERENCES

Botham, Christine M., Conning, D. M., Hayes, Jo, Litchfield, M. H. & McElligott, T. F. (1970). Effects of butylated hydroxytoluene on the enzyme activity and ultrastructure of rat hepatocytes. *Fd Cosmet. Toxicol.* **8**, 1.

Brown, W. D., Johnson, A. R. & O'Halloran, M. W. (1959). The effect of the level of dietary fat on the toxicity of phenolic antioxidants. *Aust. J. exp. Biol. med. Sci.* **37**, 533.

Clapp, N. K. (1973). An Atlas of RF Mouse Pathology: Disease Descriptions and Incidences. TID-26373. Available from: National Technical Information Service, US Department of Commerce, 5285 Port Royal Road, Springfield, VA 22151.

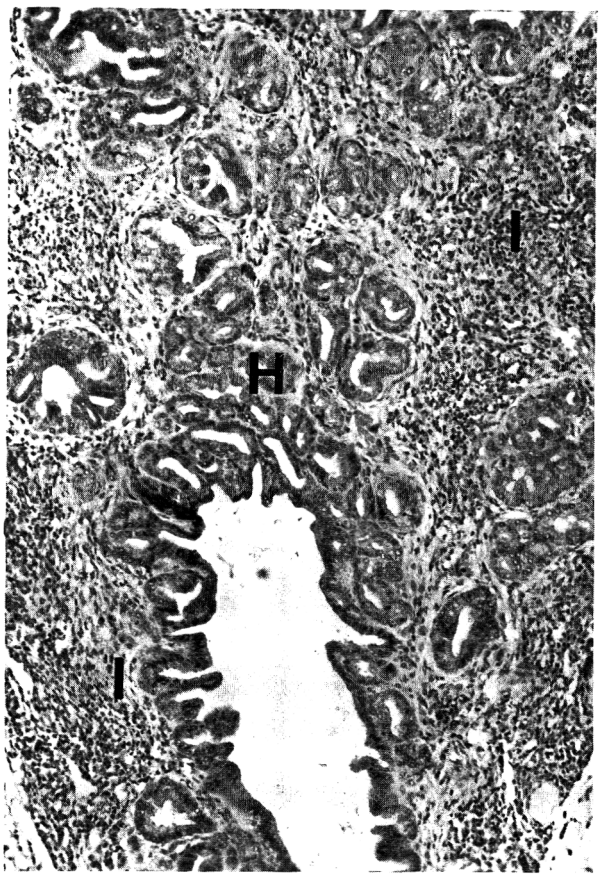

FIG. 1. Marked hyperplasia (H) of hepatic bile-duct epithelium with finger-like projections in a BHT-treated mouse. Subacute cholangitis (I) surrounds the proliferating bile ducts. Haematoxylin and eosin × 90.

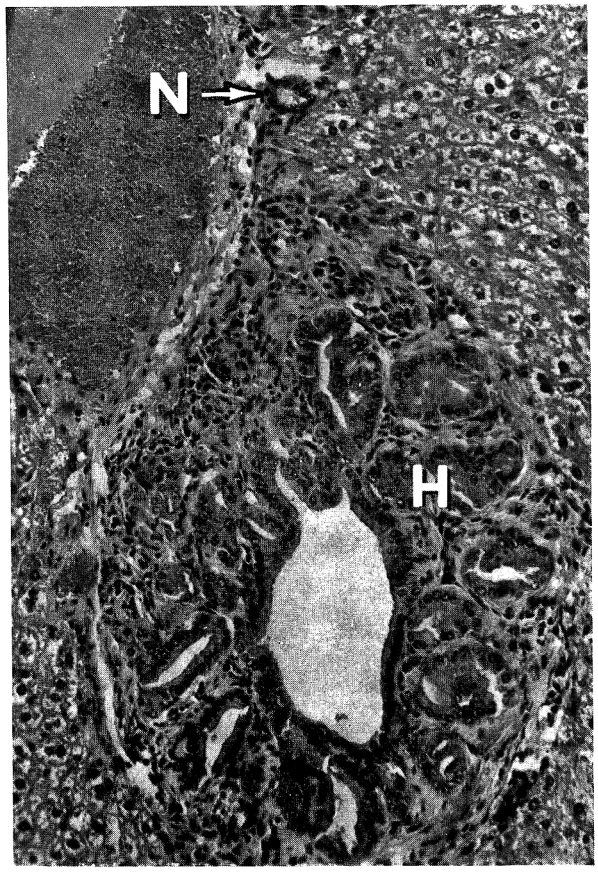

FIG. 2. Marked hyperplasia (H) of the bile-duct epithelium with cholangis and miltid fibroblastic activity with collagen formation. A normal bile duct (N) is seen for comparison. Haematoxylin and eosin × 125.

Clapp, N. K., Craig, A. W. & Toya, R. E., Sr. (1968). Oncogenicity by methyl methanesulfonate in male RF mice. *Science, N.Y.* **161,** 913.

Clapp, N. K., Craig, A. W. & Toya, R. E., Sr. (1970). Diethylnitrosamine oncogenesis in RF mice as influenced by variations in cumulative dose. *Int. J. Cancer* **5,** 119.

Clapp, N. K., Darden, E. B., Jr. & Jernigan, M. C. (1973). Relative Effects of Whole-body Sublethal Doses of 60-MeV Protons and 300-kVp X Rays on Disease Incidences in RF Mice. Radiation Research, in press.

Clapp, N. K. & Toya, R. E., Sr. (1970). Effect of cumulative dose and dose rate on dimethylnitrosamine oncogenesis in RF mice. *J. natn. Cancer Inst.* **45,** 495.

Clapp, N. K., Tyndall, R. L. & Otten, J. A. (1971). Differences in tumor types and organ susceptibility to BALB/c and RF mice following dimethylnitrosamine and diethylnitrosamine. *Cancer Res.* **31,** 196.

Creaven, P. J., Davies, W. H. & Williams, R. T. (1966). The effect of butylated hydroxytoluene, butylated hydroxyanisole and octyl gallate upon liver weight and biphenyl 4-hydroxylase activity in the rat. *J. Pharm. Pharmac.* **18,** 485.

Cumming, R. B. & Walton, M. F. (1973). Modification of acute toxicity of mutagenic and carcinogenic chemicals in the mouse by antioxidant prefeeding. *Fd Cosmet. Toxicol.* **11,** 547.

Day, A. J., Johnson, A. R., O'Halloran, M. W. & Schwartz, C. J. (1959). The effect of the antioxidant butylated hydroxy toluene on serum lipid and glycoprotein levels in the rat. *Aust. J. exp. Biol. med. Sci.* **37,** 295.

Deichmann, W. B., Clemmer, J. J., Rakoczy, R. & Bianchine, J. (1955). Toxicity of ditertiarybutylmethylphenol. *A.M.A. Archs ind. Hlth* **11,** 93.

Feuer, G., Gaunt, I. F., Golberg, L. & Fairweather, F. A. (1965). Liver response tests. VI. Application to a comparative study of food antioxidants and hepatotoxic agents. *Fd Cosmet. Toxicol.* **3,** 457.

Frawley, J. P., Kohn, F. E., Kay, J. H. & Calandra, J. C. (1965). Progress report on multigeneration reproduction studies in rats fed butylated hydroxytoluene (BHT). *Fd Cosmet. Toxicol.* **3,** 377.

Gaunt, I. F., Gilbert, D. & Martin, D. (1965). Liver response tests. V. Effect of dietary restriction on a short-term feeding study with butylated hydroxytoluene (BHT). *Fd Cosmet. Toxicol.* **3,** 445.

Gilbert, D. & Golberg, L. (1965). Liver response tests. III. Liver enlargement and stimulation of microsomal processing enzyme activity. *Fd Cosmet. Toxicol.* **3,** 417.

Gilbert, D. & Golberg, L. (1967). BHT oxidase. A liver-microsomal enzyme induced by the treatment of rats with butylated hydroxytoluene. *Fd Cosmet. Toxicol.* **5,** 481.

Gilbert, D., Martin, A. D., Gangolli, S. D., Abraham, R. & Golberg, L. (1969). The effect of substituted phenols on liver weights and liver enzymes in the rat: Structure-activity relationships. *Fd Cosmet. Toxicol.* **7,** 603.

Johnson, A. R. & Hewgill, F. R. (1961). The effect of the antioxidants, butylated hydroxy anisole, butylated hydroxy toluene and propyl gallate on growth, liver and serum lipids and serum sodium levels of the rat. *Aust. J. exp. Biol. med. Sci.* **39,** 353.

Kerr, R., Lefevre, A., Lane, B. P. & Lieber, C. S. (1966). Liver growth induced by butylated hydroxytoluene (BHT). *Clin. Res.* **14,** 299.

Lane, B. P. & Lieber, C. S. (1967). Effects of butylated hydroxytoluene on the ultrastructure of rat hepatocytes. *Lab. Invest.* **16,** 342.

Nievel, J. G. (1969). Effect of coumarin, BHT and phenobarbitone on protein synthesis in the rat liver. *Fd Cosmet. Toxicol.* **7,** 621.

Fd Cosmet. Toxicol. Vol. 11, pp. 851–854. Pergamon Press 1973. Printed in Great Britain

The Effect of pH on Dimethylnitrosamine Formation in Human Gastric Juice

R. P. LANE and M. E. BAILEY

Department of Food Science and Nutrition, University of Missouri, Columbia, 65201, USA

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Summary—In view of the possibility of additional reaction mechanisms involving nitrosyl chloride and thiocyanate ion in the nitrosation of dimethylamine in human gastric juice and the limited amount of data concerning nitrosamine formation in gastric juice, the formation of dimethylnitrosamine (DMNA) from 100 ppm nitrite and 100 ppm dimethylamine was studied in a 50% aqueous solution of human gastric juice over a pH range of 1·7–4·5. Steam distillation and extraction with methylene chloride enabled an 80% recovery at the 25 ppb ($b = 10^9$) level, with verification by high resolution mass spectrometry. DMNA was formed over the entire pH range tested in amounts ranging from 9 to 34 ppb. From the data presented here it appears that the optimum pH for the nitrosation of dimethylamine in human gastric juice is in the region of 2·5 rather than 3·4, which was previously found to be the optimum pH in aqueous solutions free of HCl.

Introduction

The nitrosation reaction of dimethylamine and nitrous acid has been studied in aqueous solutions (Mirvish, 1970; Taylor & Price, 1929). The latter paper reports an attempt to determine the reaction mechanisms involved and the possible role this reaction might play in human carcinogenesis. *N*-Nitrosamines, the products of the nitrosation of secondary amines, have been identified in several human foods (Crosby, Foreman, Palframan & Sawyer, 1972; Fazio, Damico, Howard, White & Watts, 1971; Fazio, White & Howard, 1971; Wasserman, Fiddler, Doerr, Osman & Dooley, 1972) and limited studies have shown that various *N*-nitrosamines can be formed in gastric juices both *in vitro* and *in vivo* if extremely large amounts of the secondary amine and nitrite are present (Greenblatt, Mirvish & So, 1971; Sen, Smith & Schwinghamer, 1969). It has been suggested that nitrosamines can be formed *in vivo* during the gastric digestion of cured meat when quantities of residual nitrite are high. The formation of dimethylnitrosamine (DMNA) has not been demonstrated in human gastric juice, nor has the formation of any *N*-nitrosamine been confirmed in human gastric juice from nitrite when levels used were comparable to the levels at present permitted in meat processing.

Under existing federal regulations, the use of nitrite is limited to a maximum of 200 ppm nitrite in the finished product, and for most cured meat products the level of residual nitrite is less than 100 ppm (Kolari & Aunan, 1972). There are sources of nitrite other than cured meats: the nitrite in saliva (Tannenbaum, 1972) and the nitrite formed by the bacterial reduction of nitrates, make a major contribution to the human nitrite load. This paper describes the extraction and analytical procedure for the determination of DMNA formed during the reaction for 1 hr of a solution containing 200 ppm nitrite and 200 ppm dimethylamine with an equal volume of human gastric juice at pH 1·7, 2·5, 3·5 or 4·5. The results

shown here are preliminary results of a general study of the factors affecting the *in vivo* nitrosation of dimethylamine currently being conducted in this laboratory. The final combined results will be presented upon completion of the study.

Experimental

Materials. Samples of human gastric juice were collected by stomach tube from normal fasting male and female medical students, placed on ice as soon as possible, filtered and stored at −10°C in individual brown-glass bottles. The pH of most of the gastric juices ranged from 1 to 3. By pooling samples of similar pH, 50-ml aliquots of gastric juice were obtained. To obtain the higher pH solutions, the gastric juices with the highest pH were pooled and made more basic with 0·1 N-sodium hydroxide. The 50-ml gastric-juice aliquots were mixed with water to a volume of about 90 ml, the pH being adjusted if necessary to give solutions of pH 1·7, 2·5, 3·5 and 4·5, and were then placed in a water bath at 37°C for approximately 5 min, so that all samples were equilibrated to the same temperature. Following addition of 1 ml 1% sodium nitrite and 1 ml 1% dimethylamine hydrochloride, the mixture was finally diluted to 100 ml with water, to give a final concentration of 100 ppm sodium nitrite and 100 ppm dimethylamine. The reaction mixtures were incubated in 250-ml brown-glass bottles for 1 hr at 37°C with gentle shaking, before extraction and analysis.

Extraction. The details of the extraction procedure used have been described by Lane & Bailey, 1973. Briefly this involved steam distillation from alkaline solution, followed by extraction with methylene chloride and concentration of the extract. A clean-up on an acidic Celite column, as described by Howard, Fazio & Watts (1970), was used to remove interfering compounds. Diethylnitrosamine was added as an internal standard prior to final concentration to 1 ml.

Chromatography and mass spectrometry conditions. The gas–liquid chromatograph used was an F & M model 402 equipped with an alkali flame detector (KCL-platinum iridium coil) described by Howard *et al.* (1970), and a 3·0 m × 4 mm ID glass column packed with 10% Carbowax 1540 plus 3% KOH on 100–120 mesh Chromosorb P. Flow rates were hydrogen 44, nitrogen 40 and air 275 ml/min, the hydrogen flow being adjusted periodically to give a background current of 3×10^{-9} amps. Injection, detector and column temperatures were 185, 230 and 140°C isothermal, respectively. Apparent DMNA peaks were verified using high resolution mass spectrometry with peak matching facilities and a resolution of 1/12,000, monitoring the ion peak m/e 74·0480 as described by Lane & Bailey (1973).

Thiocyanate analysis. Gastric-juice samples were analysed qualitatively for thiocyanate by the method of Koche & Hanke (1948). This procedure was adapted for quantitation by the use of standard concentrations of thiocyanate ranging from 0·25 to 2·5 mg/100 ml and colorimetric analysis before and after destruction of chromogen with silver chloride.

Results and Discussion

Three recovery runs indicated a recovery of approximately 80% for 2·5–5·0 μg DMNA added to 100 ml of diluted gastric (1:1) juice. Results of analyses of the extracts of gastric juice reaction mixtures containing nitrite and dimethylamine at pH 1·7, 2·5, 3·5 and 4·5 are shown in Fig. 1. Two samples at pH 1·7 were analysed but one was a gastric juice of higher pH, acidified with dilute HCl to pH 1·7. The results from the two samples were essentially the same.

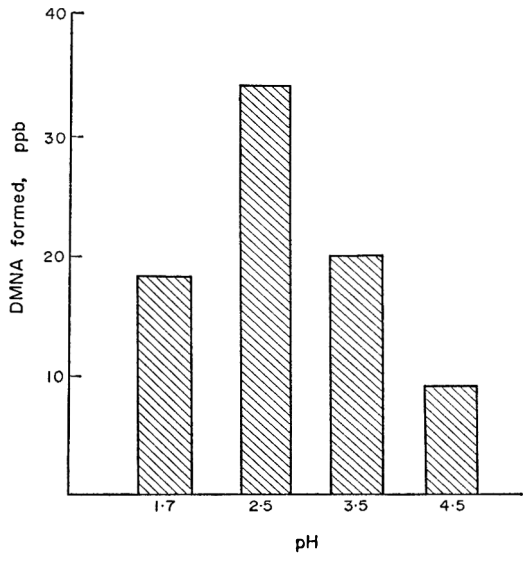

FIG. 1. Effect of pH on the formation of DMNA from nitrite and dimethylamine at levels of 100 ppm in human gastric juice at 37°C.

These results indicate that DMNA can be formed in human gastric juice from levels of nitrite and dimethylamine as low as 100 ppm. This does not mean that DMNA will necessarily be formed during human digestion from foods containing this level of nitrite. The limited data concerning the secondary amine content of foods and the variety of side reactions possible in gastric digestion make it difficult to extrapolate these results to the digestion of foods containing small quantities of nitrite. It does appear from these preliminary results that the optimum pH for this reaction may be lower than 3·4, which has been shown to be the optimum pH for the nitrosation of dimethylamine involving the nitrosyl anhydride mechanism (Mirvish, 1970). Likewise, the quantity of DMNA formed is greater than would be expected from the rate constants for this reaction in aqueous solutions containing no hydrochloric acid (Mirvish, 1970). This may be interpreted to mean that the nitrosyl chloride formed from nitrite and the hydrochloric acid in human gastric juice, though not considered of major importance at a pH above 1 (Ridd, 1961), may be contributing to the overall reaction at pH 1–3.

It has been known for some time that hydrochloric acid can catalyse nitrosation reactions (Ridd, 1961). In addition, it has been shown that small quantities of thiocyanate ion can greatly increase the reaction (Boyland, Nice & Williams, 1971). Thiocyanate at levels ranging from 1 to 4 mg/100 ml has been found in normal human gastric juice (Boxer & Rickards, 1952), and it is possible that it can catalyse the nitrosation reaction at gastric pH.

Two gastric juice samples (pH 1·7 and 2·5) pooled from several donors contained approximately 1 mg thiocyanate/100 ml. This could possibly account for the shift in optimum pH indicated by Fan & Tannenbaum (1973) working with a model system.

From these data, it appears that additional reaction mechanisms involving nitrosyl chloride and/or nitrosyl thiocyanate intermediates catalyse the nitrosation of dimethylamine in human gastric juice. This catalysis results in an increase in the formation of DMNA and

a shifting of the optimum pH to approximately 2·5. These data represent preliminary findings, but clearly suggest the need for additional research on nitrosation reactions in human gastric juice and on the activities of various catalysts that may be present during digestion.

REFERENCES

Boxer, G. E. & Rickards, J. C. (1952). Determination of thiocyanate in body fluids. *Archs Biochem. Biophys.* **39,** 292.

Boyland, E., Nice, E. & Williams, K. (1971). The catalysis of nitrosation by thiocyanate from saliva. *Fd Cosmet. Toxicol.* **9,** 639.

Crosby, N. T., Foreman, J. K., Palframan, J. F. & Sawyer, R. (1972). Estimation of steam-volatile N-nitrosamines in foods at the 1 μg/kg level. *Nature, Lond.* **238,** 342.

Fan, T.-Y. & Tannenbaum, S. R. (1973). Factors influencing the rate of formation of nitrosomorpholine from morpholine and nitrite: Acceleration by thiocyanate and other anions. *J. agric. Fd Chem.* **21,** 237.

Fazio, T., Damico, J. N., Howard, J. W., White, R. H. & Watts, J. O. (1971). Gas chromatographic determination and mass spectrometric confirmation of *N*-nitrosodimethylamine in smoke-processed marine fish. *J. agric. Fd Chem.* **19,** 250.

Fazio, T., White, R. H. & Howard, J. W. (1971). Analysis of nitrite- and/or nitrate-processed meats for N-nitrosodimethylamine. *J. Ass. off. analyt. Chem.* **54,** 1157.

Greenblatt, M., Mirvish, S. & So, B. T. (1971). Nitrosamine studies: Induction of lung adenomas by concurrent administration of sodium nitrite and secondary amines in Swiss mice. *J. natn. Cancer Inst.* **46,** 1029.

Howard, J. W., Fazio, T. & Watts, J. O. (1970). Extraction and gas chromatographic determination of N-nitrosodimethylamine in smoked fish: Application of smoked nitrite-treated chub. *J. Ass. off. analyt. Chem.* **53,** 269.

Koche, F. C. & Hanke, M. E. (1948). *Practical Methods in Biochemistry.* 5th ed. p. 124. The Williams & Wilkins Co., Baltimore.

Kolari, O. E. & Aunan, W. J. (1972). The residual levels of nitrite in cured meat products. Proceedings of 18th Meeting of Meat Research Workers, Vol. II, p. 422.

Lane, R. P. & Bailey, M. E. (1974). Procedure for the gas chromatographic determination and mass spectrometric confirmation of N-nitrosodimethylamine formed in human gastric juice. *J. agric. Fd Chem.* **22.**

Mirvish, S. S. (1970). Kinetics of dimethylamine nitrosation in relation to nitrosamine carcinogenesis. *J. natn. Cancer Inst.* **44,** 633.

Ridd, J. H. (1961). Nitrosation, diazotisation and deamination. *Q. Rev. chem. Soc.* **15,** 418.

Sen, N. P., Smith, Dorothy C. & Schwinghamer, L. (1969). Formation of *N*-nitrosamines from secondary amines and nitrite in human and animal gastric juice. *Fd Cosmet. Toxicol.* **7,** 301.

Tannenbaum, S. R. (1972). Nitrite and Nitrosamine Content of Foods: Unsolved problems and current research. Proceedings of 25th Reciprocal Meat Conference (AMSA), p. 96.

Taylor, T. W. J. & Price, L. S. (1929). The action of nitrous acid on amino compounds. Part III. Dimethylamine, N-propylamine and glycine ethyl ester. *J. chem. Soc.* p. 1897.

Wasserman, A. E., Fiddler, W., Doerr, R. C., Osman, S. F. & Dooley, C. J. (1972). Dimethylnitrosamine in frankfurters. *Fd Cosmet. Toxicol.* **10,** 681.

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MONOGRAPHS

Monographs on Fragrance Raw Materials*

D. L. J. OPDYKE

Research Institute for Fragrance Materials Inc., P.O. Box 1152, Englewood Cliffs, New Jersey 07632, USA

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AMYL CINNAMIC ALDEHYDE

Synonyms: α-Amyl cinnamic aldehyde; α-*n*-amyl-β-phenylacrolein.
Structure: $C_6H_5 \cdot CH{:}C(CH_2 \cdot [CH_2]_3 \cdot CH_3) \cdot CHO$.
Description and physical properties: EOA Spec. no. 45.
Occurrence: Apparently has not been reported to occur in nature.
Preparation: By the condensation of benzaldehyde with heptaldehyde, usually in an alkaline medium (Bedoukian, 1967).
Uses: In public use since the 1920s. Use in fragrances in the USA amounts to about 800,000 lb/yr.

Concentration in final product (%):

	Soap	*Detergent*	*Creams, lotions*	*Perfume*
Usual	0·04	0·004	0·015	0·36
Maximum	0·40	0·03	0·05	1·2

Analytical data: Gas chromatogram, RIFM no. 70–22; infra-red curve, RIFM no. 70–22.

Status

Amyl cinnamic aldehyde 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 amyl cinnamic aldehyde (α-amyl cinnamaldehyde), in the list of admissible artificial flavouring substances at a level of 1 ppm. The *Food Chemicals Codex* (1972) has a monograph on amyl cinnamic aldehyde.

Biological data

Acute toxicity. The acute oral LD_{50} value was reported as 3·73 g/kg in the rat (Jenner, Hagan, Taylor, Cook & Fitzhugh, 1964) and the acute dermal LD_{50} in rabbits as >2 g/kg (Moreno, 1973).

Human testing. A maximization test (Kligman, 1966) was carried out on 25 volunteers. The

*The second set of these monographs was published in *Food and Cosmetics Toxicology* (1973, **11**, 477).

material was tested at 6% concentration in petrolatum and produced no reactions (Greif, (1967).

Metabolism. So far as is known, all aromatic aldehydes are metabolized in the animal body by oxidation to the corresponding acids. In some instances, the aldehydes are excreted as glucuronides. Cinnamic aldehyde is oxidized to cinnamic acid which is then degraded to benzoic acid, but ethyl cinnamic aldehyde is oxidized to the corresponding acid and is not further metabolized (Williams, 1959).

References

Bedoukian, P. Z. (1967). *Perfumery and Flavoring Synthetics.* 2nd ed. p. 27. Elsevier Publishing Co., New York.

Council of Europe (1970). Natural and Artificial Flavouring Substances. Partial Agreement in the Social and Public Health Field. List A(1), Series 1, no. 128, p. 55. Strasbourg.

Flavoring Extract Manufacturers' Association (1965). Survey of flavoring ingredient usage levels. No. 2061. *Fd Technol., Champaign* **19** (2), part 2, 155.

Food Chemicals Codex (1972). 2nd ed. Prepared by the Committee on Specifications, Food Chemicals Codex, of the Committee on Food Protection. p. 53. National Academy of Sciences–National Research Council Publ. 1406, Washington, D.C.

Greif, N. (1967). Cutaneous safety of fragrance materials as measured by the maximization test. *Am. Perfumer Cosmet.* **82** (June), 54.

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.

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

Moreno, O. M. (1973). Report to RIFM, 1 February.

Williams, R. T. (1959). *Detoxication Mechanisms. The Metabolism and Detoxication of Drugs, Toxic Substances and Other Organic Compounds.* 2nd ed. p. 332. Chapman & Hall Ltd., London.

AMYL CINNAMIC ALDEHYDE DIETHYL ACETAL

Synonyms: 1,1-Diethoxy-2-amyl-3-phenyl-2-propene; 1,1-diethoxyl-2-*n*-amyl-3-phenylacrolein.

Structure: $C_6H_5 \cdot CH{:}C(CH_2 \cdot [CH_2]_3 \cdot CH_3) \cdot CH(OC_2H_5)_2$.

Description and physical properties: Almost colourless oily liquid with a leafy floral odour (Arctander, 1969).

Occurrence: Apparently has not been reported to occur in nature.

Preparation: From amyl cinnamic aldehyde and ethyl alcohol by condensation (Arctander, 1969).

Uses: In public use since the 1950s. Use in fragrances in the USA amounts to less than 3000 lb/yr.

Concentration in final product (%):

	Soap	*Detergent*	*Creams, lotions*	*Perfume*
Usual	—	—	0·015	0·036
Maximum	—	—	0·05	1·2

Analytical data: Gas chromatogram, RIFM no. 71–26; infra-red curve, RIFM no. 71–26.

Biological data

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

Irritation. Amyl cinnamic aldehyde diethyl acetal tested at 10% in petrolatum produced no irritation after a 48-hr closed patch test in 25 human subjects (Kligman, 1971).

Human testing. 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 reactions (Kligman, 1971).

References

Arctander, S. (1969). *Perfume and Flavor Chemicals (Aroma Chemicals)*. Vol. 1, p. 154. S. Arctander, Montclair, New Jersey.

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

Kligman, A. M. (1971). Report to RIFM, 27 September.

Moreno, O. M. (1973). Report to RIFM, 9 April.

AMYL SALICYLATE

Synonyms: Isoamyl *o*-hydroxybenzoate; isoamyl salicylate.
Structure: $C_6H_4 \cdot COOC_5H_{11}$.
Description and physical properties: EOA Spec. no. 27.
Occurrence: Apparently has not been reported to occur in nature.
Preparation: By esterification of salicylic acid with the isomeric amyl alcohols obtained from fusel oil and other sources.
Uses: In public use since the 1900s. Use in fragrances in the USA amounts to about 600,000 lb/yr.

Concentration in final product (%):

	Soap	*Detergent*	*Creams, lotions*	*Perfume*
Usual	0·03	0·003	0·015	0·4
Maximum	0·30	0·03	0·05	1·2

Analytical data: Gas chromatogram, RIFM no. 70–13; infra-red curve, RIFM no. 70–13.

Status

Amyl salicylate 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 amyl salicylate (isoamyl salicylate) in the list of admissible artificial flavouring substances at a level of 3 ppm. The *Food Chemicals Codex* (1972) has a monograph on amyl salicylate.

Biological data

Acute toxicity. The LD_{50} value iv in dogs was reported as 0·5–0·8 g/kg (Fassett, 1963).

Human testing. 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 reactions (Kligman, 1970). Aspirin-containing drugs will cause exacerbation in some patients with chronic urticaria. The action is probably due to the salicylate radical. It is suggested that aspirin acts in chronic urticaria by enhancing the effect of histamine in the skin (Moore-Robinson & Warin, 1967).

Metabolism. Most of the esters of salicylic acid are decomposed to salicylic acid in the body and excreted as such. Besides the unchanged acid, salicuric acid, genticic acid and salicyl glucuronide have been known to be excreted. The lower esters of salicylic acid decompose more readily than the higher esters and as a consequence, little of the amyl ester is split. The whole subject of salicylic esters is covered in an extensive literature by Gross & Greenberg (1948).

References

Council of Europe (1970). Natural and Artificial Flavouring Substances. Partial Agreement in the Social and Public Health Field. List A(1), Series 1, no. 437, p. 72. Strasbourg.

Fassett, D. W. (1963). Esters. In *Industrial Hygiene and Toxicology*. Vol. II, 2nd ed. Edited by F. A. Patty. p. 1847. Interscience Publishers, New York.

Flavoring Extract Manufacturers' Association (1965). Survey of flavoring ingredient usage levels. No. 2084. *Fd Technol., Champaign* **19** (2), part 2, 155.

Food Chemicals Codex (1972). 2nd ed. Prepared by the Committee on Specifications, Food Chemicals Codex, of the Committee on Food Protection. p. 402. National Academy of Sciences–National Research Council Publ. 1406, Washington, D.C.

Gross, M. & Greenberg, L. A. (1948). *The Salicylates.* Hillhouse Press, New Haven, Conn.

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

Kligman, A. M. (1970). Report to RIFM, 1 June.

Moore-Robinson, Miriam & Warin, R. P. (1967). Effects of salicylates in urticaria. *Br. med. J.* **4**, 262.

Williams, R. T. (1959). *Detoxication Mechanisms. The Metabolism and Detoxication of Drugs, Toxic Substances and Other Organic Compounds.* 2nd ed. p. 363. Chapman & Hall Ltd., London.

AMYRIS OIL ACETYLATED

Description and physical properties: A yellowish liquid with a lighter and greener odour than that of amyris oil (Arctander, 1960).

Occurrence: Apparently has not been reported to occur in nature.

Preparation: By acetylation of amyris oil (Arctander, 1960).

Uses: In public use since the 1950s. Use in fragrances in the USA amounts to less than 5000 lb/yr.

Concentration in final product (%):

	Soap	*Detergent*	*Creams, lotions*	*Perfume*
Usual	0·04	0·004	0·015	0·54
Maximum	0·24	0·024	0·06	1·5

Analytical data: Gas chromatogram, RIFM no. 72-9; infra-red curve, RIFM no. 72-9.

Status

Amyris oil acetylated is not listed by the Council of Europe (1970), by the FDA or by the Food Chemicals Codex (1972), but the parent substance is.

Amyris oil is approved by the FDA for food use (21 CFR 121.1163). The Council of Europe (1970) included amyris oil (*Amyris balsamifera*) in the list of temporarily admitted flavouring substances. The *Food Chemicals Codex* (1972) has a monograph on amyris oil.

Biological data

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

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

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

References

Arctander, S. (1960). *Perfume and Flavor Materials of Natural Origin.* p. 61. S. Arctander, Elizabeth, New Jersey.

Council of Europe (1970). Natural and Artificial Flavouring Substances. Partial Agreement in the Social and Public Health Field. List N(1), Series 2, no. 33, p. 14. Strasbourg.

Food Chemicals Codex (1972). 2nd ed. Prepared by the Committee on Specifications, Food Chemicals Codex, of the Committee on Food Protection. p. 59. National Academy of Sciences–National Research Council Publ. 1406, Washington, D.C.

Keating, J. W. (1972). Report to RIFM, 20 April.

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

Kligman, A. M. (1972). Report to RIFM, 19 July.

ANETHOLE

Synonyms: *p*-Propenylphenyl methyl ether; *p*-propenylanisole.
Structure: $CH_3 \cdot CH{:}CH \cdot C_6H_4 \cdot OCH_3$. (Most commercial anethole consists of mixed isomers.)
Description and physical properties: *Food Chemicals Codex* (1972).
Occurrence: Found in oils of aniseed, star-anise, fennel and leaves of *Clausena anisata* Hook., *Backhousia anisata* and *Magnolia salicifolia* Maxim. (Givaudan, 1961).
Preparation: By isomerization of estragole using alcoholic potassium hydroxide as agent (Arctander, 1969).
Uses: In public use since the 1930s. Use in fragrances in the USA amounts to about 16,000 lb/yr.

Concentration in final product (%):

	Soap	*Detergent*	*Creams, lotions*	*Perfume*
Usual	0·008	0·001	0·0025	0·054
Maximum	0·06	0·006	0·01	0·25

Status

Anethole was granted GRAS status by FEMA (1965) and is approved by the FDA for food use (GRAS). The Council of Europe (1970) listed *trans*-anethole (propenyl anisole), giving an ADI of 1·5 mg/kg. The *Food Chemicals Codex* (1972) has a monograph on anethole and the Joint FAO/WHO Expert Committee on Food Additives (1967) has published a monograph and specifications for *trans*-anethole giving an ADI of 0–1·25 mg/kg for man.

Biological data

Acute toxicity. The acute oral LD_{50} has been reported as 2090 mg/kg in the rat, 3050 mg/kg in the mouse and 2160 mg/kg in the guinea-pig (Jenner, Hagan, Taylor, Cook & Fitzhugh, 1964). The acute dermal LD_{50} was reported as >5 g/kg in the rabbit (Hart, 1971). No percutaneous absorption of anethole occurred through the skin of a mouse within 2 hr (Meyer & Meyer, 1959).

Chronic toxicity. In a feeding study, 10,000 ppm fed to rats in the diet for 15 wk produced slight hydropic changes in the liver of the male animals (Hagan, Hansen, Fitzhugh, Jenner, Jones, Taylor, Long, Nelson & Brouwer, 1967). In other feeding studies in rats, neither 2500 ppm fed in the diet for 1 yr (Bär & Griepentrog, 1967) nor 10,000 ppm in the diet for 15 wk produced any effects (Bär & Griepentrog, 1967). The level causing no effect in the rat was 2500 ppm in the diet or 125 mg/kg body weight/day (Joint FAO/WHO Expert Committee on Food Additives, 1967). In a feeding study, in which dietary levels of 1000, 3000, 10,000 and 30,000 ppm were fed to rats for 90 days, death occurred with the highest level, and survival was affected at the 10,000 ppm level. Hepatocellular oedema, degeneration and regeneration were found, in proportion to the level fed, at 3000 ppm and above. No effect was seen at 1000 ppm (Joint FAO/WHO Expert Committee on Food Additives (1967).

Irritation. Anethole tested at 2% in petrolatum produced no irritation after a 48-hr closed patch test in 25 human subjects (Kligman, 1971).

Human testing. A maximization test (Kligman, 1966) was carried out on 25 volunteers. The material was tested at a 2% concentration in petrolatum and produced no reactions (Kligman, 1971).

Metabolism. Anethole is metabolized by oxidation of the propenyl group and is excreted as anisic acid (Williams, 1959). The metabolism of *trans*-anethole used in the preparation of anis-flavoured alcoholic beverages was studied in the rabbit and rat after iv and oral administration. It was excreted rapidly from the animal regardless of the method of administration. After iv injection it was found concentrated in the liver, lungs and brain; after oral administration, absorption was slight and most of it remained in the stomach. Ethyl alcohol has no effect on the metabolism (Le Bourhis, 1968).

References

Arctander, S. (1969). *Perfume and Flavor Chemicals (Aroma Chemicals).* Vol. 1. p. 236. S. Arctander, Montclair, New Jersey.

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

Council of Europe (1970). Natural and Artificial Flavouring Substances. Partial Agreement in the Social and Public Health Field. List A(1), Series 1, no. 184, p. 58. Strasbourg.

Flavoring Extract Manufacturers' Association (1965). Survey of flavoring ingredient usage levels. No. 2086. *Fd Technol., Champaign* **19** (2), part 2, 155.

Food Chemicals Codex (1972). 2nd ed. Prepared by the Committee on Specifications, Food Chemicals Codex, of the Committee on Food Protection. p. 56. National Academy of Sciences–National Research Council Publ. 1406, Washington, D.C.

Givaudan Index (1961). *Specifications of Synthetics and Isolates for Perfumery.* 2nd ed. p. 56. Givaudan–Delawanna, Inc., New York.

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.

Hart, E. R. (1971). Report to RIFM, 18 June.

Jenner, P. M., Hagan, E. C., Taylor, Jean M., Cook, E. L. & Fitzhugh, O. G. (1964). Food flavourings and compounds of related structure. I. Acute oral toxicity. *Fd Cosmet. Toxicol.* **2,** 327.

Joint FAO/WHO Expert Committee on Food Additives (1967). Toxicological Evaluation of Some Flavouring Substances and Non-nutritive Sweetening Agents. *F.A.O. Nutr. Mtg Rep. Ser. no.* 44A, Geneva, p. 71, WHO/Food Add./68.33.

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

Kligman, A. M. (1971). Report to RIFM, 14 June.

Le Bourhis, B. (1968). Recherches préliminaires sur le métabolisme du *trans*-anéthole. *Annls Biol. clin.* **26,** 711.

Meyer, Fr. & Meyer, E. (1959). Percutaneous absorption of essential oils and their constituents. *Arzneimittel-Forsch.* **9,** 516.

Williams, R. T. (1959). *Detoxication Mechanisms. The Metabolism and Detoxication of Drugs, Toxic Substances and Other Organic Compounds.* 2nd ed. p. 326. Chapman & Hall Ltd., London.

ANISE OIL

Description and physical properties: Food Chemicals Codex (1972). The characteristic odour of anise oil is due to its high content (80–90%) of anethole. Small quantities of methyl chavicol, *p*-methoxyacetophenone and other materials have also been reported to occur in this oil (Gildemeister & Hoffman, 1961).

Occurrence: Found in the dried ripe fruit of *Pimpinella anisum* L. (Umbelliferae) (*Merck Index*, 1968).

Preparation: By steam distillation of the dried fruits of the herb *Pimpinella anisum* L. (Umbelliferae) (Arctander, 1960).

Uses: In public use since the 1860s. Use in fragrances in the USA amounts to less than 1000 lb/yr.

Concentration in final product (%):

	Soap	*Detergent*	*Creams, lotions*	*Perfume*
Usual	0·008	0·001	0·0025	0·054
Maximum	0·06	0·006	0·01	0·25

Analytical data: Gas chromatogram, RIFM no. 71–30; infra-red curve, RIFM no. 71–30.

Status

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

Biological data

Acute toxicity. The acute oral LD_{50} in rats was reported as 2·25 (1·82–2·74) g/kg (Weir, 1971). The acute dermal LD_{50} was reported as >5 g/kg in the rabbit (Weir, 1971). No percutaneous absorption of anise oil occurred through the skin of a mouse within 2 hr (Meyer & Meyer, 1959).

Irritation. Anise oil tested at a concentration of 2% in petrolatum produced no irritation after a 48-hr closed patch test in 25 human subjects (Kligman, 1971).

Human testing. A maximization test (Kligman, 1966) was carried out on 25 volunteers. The material was tested at a 2% concentration in petrolatum and produced no reactions (Kligman, 1971). One authority states that aniseed oil is not a primary irritant to normal skin (Harry, 1948).

Sensitization. Several cases of sensitivity have been reported (Loveman, 1938; Schwartz, 1934; Tulipan, 1938). The irritating substance is anethole and the dermatitis consists of erythema, scaling and vesiculation (Schwartz, Tulipan & Peck, 1947).

Metabolism. See monograph on anethole.

References

Arctander, S. (1960). *Perfume and Flavor Materials of Natural Origin.* p. 67. S. Arctander, Elizabeth, New Jersey.

Council of Europe (1970). Natural and Artificial Flavouring Substances. Partial Agreement in the Social and Public Health Field. List N(1), Series 1(b), no. 336, p. 24. Strasbourg.

Flavoring Extract Manufacturers' Association (1965). Survey of flavoring ingredient usage levels. No. 2094. *Fd Technol., Champaign* **19** (2), part 2, 155.

Food Chemicals Codex (1972). 2nd ed. Prepared by the Committee on Specifications, Food Chemicals Codex, of the Committee on Food Protection. p. 59. National Academy of Sciences–National Research Council Publ. 1406, Washington, D.C.

Gildemeister, E. u. Hoffman, F. (1961). *Die Ätherischen Öle.* Vol. VI. p. 425. Akademie Verlag, Berlin.

Harry, R. G. (1948). *Cosmetic Materials.* Vol. II. Leonard Hill, London.

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

Kligman, A. M. (1971). Report to RIFM, 3 November.

Loveman, A. B. (1938). Stomatitis venenata: Report of a case of sensitivity of the mucous membranes and the skin to oil of anise. *Archs Derm. Syph.* **37**, 70.

Merck Index (1968). *An Encyclopedia of Chemicals and Drugs.* 8th ed. p. 756. Merck & Co., Inc., Rahway, New Jersey.

Meyer, Fr. & Meyer, E. (1959). Percutaneous absorption of essential oils and their constituents. *Arzneimittel-Forsch.* **9**, 516.

Schwartz, L. (1934). Skin Hazards in American Industry. Part I. U.S. Publ. Hlth Bull. no. 215.

Schwartz, L., Tulipan, L. & Peck, S. M. (1947). Occupational Diseases of the Skin. Lea and Febiger, Philadelphia.

Tulipan, L. (1938). Cosmetic irritants. *Arch. Derm. Syph.* **38**, 906.

Weir, R. J. (1971). Report to RIFM, 25 August.

BASIL OIL, SWEET

Description and physical properties: Food Chemicals Codex (1972). European sweet basil oil may contain about 55% methyl chavicol and 35% of alcohols calculated as linalool, along with other components (Gildemeister & Hoffman, 1961).
Occurrence: Found in the leaves of *Ocimum basilicum* L. (Labiatae) (*Merck Index*, 1968).
Preparation: By steam distillation of the flowering tops of the plant *Ocimum basilicum* L. (Labiatae) (Arctander, 1960).
Uses: In public use since the 1900s. Use in fragrances in the USA amounts to less than 2000 lb/yr.

Concentration in final product (%):

	Soap	*Detergent*	*Creams, lotions*	*Perfume*
Usual	—	—	0·0025	0·09
Maximum	—	—	0·01	0·4

Analytical data: Gas chromatogram, RIFM no. 71–33; infra-red curve, RIFM no. 71–33.

Status

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

Biological data

Acute toxicity. The acute oral LD_{50} value in the rat was reported to be 1·4 (0·56–3·5) ml/kg (Levenstein, 1972). The acute dermal LD_{50} was reported as >5 ml/kg (Levenstein, 1972).

Irritation. Basil oil applied undiluted to the backs of hairless mice produced mildly irritating effects (Urbach & Forbes, 1972). Basil oil tested at a concentration of 4% in petrolatum produced no irritation after a 48-hr closed patch test in 25 human subjects (Kligman, 1972).

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

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

References

Arctander, S. (1960). *Perfume and Flavor Materials of Natural Origin.* p. 81. S. Arctander, Elizabeth, New Jersey.

Council of Europe (1970). Natural and Artificial Flavouring Substances. Partial Agreement in the Social and Public Health Field. List N(1), Series 1(b), no. 308, p. 23. Strasbourg.

Flavoring Extract Manufacturers' Association (1965). Survey of flavoring ingredient usage levels. No. 2118. *Fd Technol., Champaign* **19** (2), part 2, 155.

Food Chemicals Codex (1972). 2nd ed. Prepared by the Committee on Specifications, Food Chemicals Codex, of the Committee on Food Protection. p. 73. National Academy of Sciences–National Research Council Publ. 1406, Washington, D.C.

Gildemeister, E. u. Hoffman, F. (1961). *Die Ätherischen Öle.* Vol. VII. p. 478. Akademie Verlag, Berlin.

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

Kligman, A. M. (1972). Report to RIFM, 14 March.

Levenstein, I. (1972). Report to RIFM, 13 January.

Merck Index (1968). *An Encyclopedia of Chemicals and Drugs.* 8th ed. p. 756. Merck & Co., Inc., Rahway, New Jersey.

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

BAY OIL

Description and physical properties: EOA Spec. no. 251. The oil consists mainly of eugenol and chavicol (55–65%). The major portion of the balance consists of terpenes (α-pinene, myrcene and dipentene). Small quantities of citral, nerol, cineol and other terpenoids have also been identified in bay oil (Gildemeister & Hoffman, 1961).
Occurrence: Found in the leaves of the bay tree, *Pimenta racemosa* (Miller) J. W. Moore (Myrtaceae).
Preparation: By steam distillation of the leaves of the bay tree, *Pimenta racemosa* (Miller) J. W. Moore (Myrtaceae).
Uses: In public use since the 1860s. Use in fragrances in the USA amounts to less than 10,000 lb/yr.

Concentration in final product (%):

	Soap	*Detergent*	*Creams, lotions*	*Perfume*
Usual	0·01	0·001	0·0025	0·09
Maximum	0·09	0·009	0·01	1·5

Analytical data: Gas chromatogram, RIFM no. 71–34; infra-red curve, RIFM no. 71–34.

Status

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

Biological data

Acute toxicity. The acute oral LD_{50} value in the rat was reported as 1800 (1406–2304) mg/kg (Owen, 1971a). The acute dermal LD_{50} was reported as >5 ml/kg in the rabbit (Owen, 1971b).

Irritation. Bay oil tested at a concentration of 10% in petrolatum produced no irritation after a 48-hr closed patch test in 25 human subjects (Kligman, 1971).

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

References

Council of Europe (1970). Natural and Artificial Flavouring Substances. Partial Agreement in the Social and Public Health Field. List N(1), Series 1(b), no. 334, p. 24. Strasbourg.

Flavoring Extract Manufacturers' Association (1965). Survey of flavoring ingredient usage levels. No. 2125. *Fd Technol., Champaign* **19** (2), part 2, 155.

Food Chemicals Codex (1972). 2nd ed. Prepared by the Committee on Specifications, Food Chemicals Codex, of the Committee on Food Protection. p. 74. National Academy of Sciences–National Research Council Publ. 1406, Washington, D.C.

Gildemeister, E. u. Hoffman, F. (1961). *Die Ätherischen Öle.* Vol. VI, p. 83. Akademie Verlag, Berlin.
Kligman, A. M. (1966). The identification of contact allergens by human assay. III. The maximization test. A procedure for screening and rating contact sensitizers. *J. invest. Derm.* **47,** 393.
Kligman, A. M. (1971). Report to RIFM, 9 June.
Owen, G. (1971a). Report to RIFM, 28 June.
Owen, G. (1971b). Report to RIFM, 1 July.

BENZOIN (RESINOID)

Description and physical properties: An amber coloured resinoid with a characteristic balsamic odour. Both Siam and Sumatra benzoins contain vanillin and esters of benzoic and cinnamic acids (Naves & Mazuyer, 1947).

Occurrence: Benzoin Siam is obtained from *Styrax tonkinensis* P. *S. mycrothyrsus* P. Benzoin Sumatra is derived from *Styrax benzoin* D. *S. benzoides* C. (Naves & Mazuyer, 1947).

Preparation: The resinoid is obtained by extracting the natural benzoin with benzene and then distilling off the solvent (Naves & Mazuyer, 1947).

Uses: In public use since the 1900s. Use in fragrances in the USA amounts to about 100,000 lb/yr.

Concentration in final product (%):

	Soap	*Detergent*	*Creams, lotions*	*Perfume*
Usual	0·03	0·003	0·0075	0·27
Maximum	0·21	0·002	0·03	0·8

Analytical data: Infra-red curve, RIFM no. 70–8.

Status

Benzoin 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 benzoin in the list of substances, spices and seasonings whose use it deemed admissible with a possible limitation of the active principle in the final product. The *United States Pharmacopeia* (1965) has a monograph on benzoin.

Biological data

Acute toxicity. The acute oral LD_{50} was reported as 10 g/kg in the rat (Margolin, 1970a). The acute dermal LD_{50} in the rabbit was reported as 8·87 (3·98–19·75) g/kg (Margolin, 1970b).

Human testing. 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 reactions (Kligman, 1970).

Sensitization. Numerous cases of compound tincture of benzoin sensitivity have been reported in the literature, with eczema as the major dermatological manifestation (Spott & Shelley, 1970). There have been cross-sensitization reactions to benzoin when subjects were sensitized to Balsam Peru (Hjorth, 1961). Benzoin in the concentrations and forms employed in toilet preparations and dermatological preparations is not a primary irritant and there is no evidence that it is a sensitizer (Harry, 1948). Dermatitis has been reported from the use of a Compound Tincture of Benzoin, but this contains other substances such as Tolu, Balsam Peru and styrax, to which some persons are known to be sensitized (James, 1930).

References

Council of Europe (1970). Natural and Artificial Flavouring Substances. Partial Agreement in the Social and Public Health Field. List N(1), Series 1(b), no. 439, p. 28. Strasbourg.

Flavoring Extract Manufacturers' Association (1965). Survey of flavoring ingredient usage levels. No. 2133. *Fd Technol., Champaign* **19** (2), part 2, 155.

Harry, R. G. (1948). *Cosmetic Materials.* Vol. II. p. 71. Leonard Hill, London.
Hjorth, N. (1961). *Eczematous Allergy To Balsams.* p. 80. Aarhuus Stiftsbogtrykkerie. Munksgaard, Copenhagen.
James, B. M. (1930). Dermatitis produced by compound tincture of benzoin. *J. med. Soc. New Jers.* **27**, 596.
Kligman, A. M. (1966). The identification of contact allergens by human assay. III. The maximization test. A procedure for screening and rating contact sensitizers. *J. invest. Derm.* **47**, 393.
Kligman, A. M. (1970). Report to RIFM, 7 October.
Margolin, S. (1970a). Report to RIFM, 1 July.
Margolin, S. (1970b). Report to RIFM, 12 October.
Naves, Y. R. & Mazuyer, G. (1947). *Natural Perfume Materials.* Reinholt, New York.
Spott, D. A. & Shelley, W. B. (1970). Exanthem due to contact allergen (benzoin) absorbed through skin. *J. Am. med. Ass.* **214** (10), 1881.
United States Pharmacopeia (1965). 17th revision. Prepared by the Committee of Revision. p. 71. New York.

BENZOPHENONE

Synonyms: Diphenyl ketone; benzoylbenzene.
Structure: $C_6H_5 \cdot CO \cdot C_6H_5$.
Description and physical properties: EOA Spec. no. 83.
Occurrence: Apparently has not been reported to occur in nature.
Preparation: By standard Friedel–Craft type reactions (Bedoukian, 1967).
Uses: In public use since the 1920s. Use in fragrances in the USA amounts to about 100,000 lb/yr.

Concentration in final product (%):

	Soap	*Detergent*	*Creams, lotions*	*Perfume*
Usual	0·02	0·002	0·004	0·09
Maximum	0·15	0·015	0·015	0·3

Status

Benzophenone 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 benzophenone in the list of admissible artificial flavouring substances at a level of 2 ppm. The *Food Chemicals Codex* (1972) has a monograph on benzophenone.

Biological data

Acute toxicity. The acute oral LD_{50} was reported as > 10,000 mg/kg in the rat (Margolin, 1970a). The acute dermal LD_{50} was reported to be 3535 (2007–6226) mg/kg in the rabbit (Margolin, 1970b).

Human testing. A maximization test (Kligman, 1966) was carried out on 25 volunteers. The material was tested at a 6% concentration in petrolatum and produced no reactions (Kligman, 1970).

Sensitization. Because of their extensive use as sunscreens (Knox, Guin & Cockerell, 1958) substituted benzophenones have been reported as capable of producing allergic reactions, but even here the incidence as reported by the authors "is extremely low" (Ramsay, Cohen & Baer, 1972).

Metabolism. Benzophenone's main metabolic pathway in the rabbit is by reduction to benzhydrol, which is excreted conjugated with glucuronic acid (Williams, 1959).

References

Bedoukian, P. Z. (1967). *Perfumery and Flavoring Synthetics.* 2nd ed. p. 154. Elsevier Publishing Co., New York.

Council of Europe (1970). Natural and Artificial Flavouring Substances. Partial Agreement in the Social and Public Health Field. List A(1), Series 1, no. 167, p. 57. Strasbourg.

Flavoring Extract Manufacturers' Association (1965). Survey of flavoring ingredient usage levels. No. 2134. *Fd Technol., Champaign* **19** (2), part 2, 155.

Food Chemicals Codex (1972). 2nd ed. Prepared by the Committee on Specifications, Food Chemicals Codex, of the Committee on Food Protection. p. 81. National Academy of Sciences–National Research Council Publ. 1406, Washington, D.C.

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

Kligman, A. M. (1970). Report to RIFM, 1 June.
Knox, A. M., Guin, J. & Cockerell, E. (1958). Benzophenones. Ultraviolet light-absorbing agents. *J. invest. Derm.* **30,** 295.
Margolin, S. (1970a). Report to RIFM, 1 July.
Margolin, S. (1970b). Report to RIFM, 12 October.
Ramsay, D. L., Cohen, H. J. & Baer, R. L. (1972). Allergic reaction to benzophenone. Simultaneous occurrence of urticarial and contact sensitivities. *Archs Derm.* **105,** 906.
Williams, R. T. (1959). *Detoxication Mechanisms. The Metabolism and Detoxication of Drugs, Toxic Substances and Other Organic Compounds.* 2nd ed. p. 336. Chapman & Hall Ltd., London.

BENZYL ACETATE

Structure: $C_6H_5 \cdot CH_2 \cdot OCOCH_3$.
Description and physical properties: EOA Spec. no. 33.
Occurrence: Found in a dozen essential oils including jasmin, hyacinth and gardenia (Gildemeister & Hoffman, 1960).
Preparation: By the interaction of benzyl chloride and sodium acetate or by acetylation of benzyl alcohol.
Uses: In public use since the 1900s. Use in fragrances in the USA amounts to less than 1,000,000 lb/yr.

Concentration in final product (%):

	Soap	*Detergent*	*Creams, lotions*	*Perfume*
Usual	0·05	0·005	0·015	0·54
Maximum	0·36	0·036	0·15	3·0

Analytical data: Gas chromatogram, RIFM no. 70–63; infra-red curve, RIFM no. 70–63.

Status

Benzyl acetate 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 benzyl acetate, giving an ADI of 5 mg/kg. The *Food Chemicals Codex* (1972) has a monograph on benzyl acetate, and the Joint FAO/WHO Expert Committee on Food Additives (1967) has published a monograph and specification for benzyl acetate, giving an unconditional ADI of 0·5 mg/kg body weight in man.

Biological data

Acute toxicity. The acute oral LD_{50} in rats was reported as 2·49 g/kg by Jenner, Hagan, Taylor, Cook & Fitzhugh (1964) and as 3·69 g/kg by Boyd & Kuizenga (1945). The LD_{50} by dermal application was reported as > 5 g/kg in the rabbit (Moreno, 1972). Benzyl acetate caused hyperaemia of the lungs and moderate pulmonary oedema in mice that died from exposure to the vapour of the ester (von Oettingen, 1960).

Human testing. 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 reactions (Greif, 1967).

Inhalation effects. The vapour of benzyl acetate is irritating to the eyes and to respiratory passages, exciting cough (Lehmann & Flury, 1943). Benzyl acetate is absorbed through the lungs and from the gastro-intestinal tract and its vapours have an irritating effect on the mucous membranes (von Oettingen, 1960). The threshold limit value for benzyl acetate has been set at 15 ppm, at which level it has an irritant and narcotic effect (*Handbook of Organic Industrial Solvents*, 1961). Both cyclohexyl and benzyl acetates appear to produce narcosis and lethal effects in animals at levels considerably below those of the other esters. They are of low volatility, however, and except for local irritation no effects have been reported in man (Fassett, 1963).

Metabolism. The esters of benzyl alcohol, such as the acetate, benzoate, cinnamate and hydrocinnamate, are rapidly hydrolysed *in vivo* to benzyl alcohol which is then oxidized to benzoic acid and excreted as hippuric acid (Williams, 1959).

References

Boyd, G. & Kuizenga, M. (1945). Toxicity studies of benzyl benzoate and related benzyl compounds. *J. Parmacol.* **84,** 358.

Council of Europe (1970). Natural and Artificial Flavouring Substances. Partial Agreement in the Social and Public Health Field. List A(1), Series 1, no. 205, p. 59. Strasbourg.

Fassett, D. W. (1963). Esters. In *Industrial Hygiene and Toxicology.* Vol. II. 2nd ed. Edited by F. A. Patty. p. 1847. Interscience Publishers, New York.

Flavoring Extract Manufacturers' Association (1965). Survey of flavoring ingredient usage levels. No. 2135. *Fd Technol., Champaign* **19** (2), part 2, 155.

Food Chemicals Codex (1972). 2nd ed. Prepared by the Committee on Specifications, Food Chemicals Codex, of the Committee on Food Protection. p. 83. National Academy of Sciences–National Research Council Publ. 1406, Washington, D.C.

Gildemeister, E. u. Hoffman, F. (1960). *Die Ätherischen Öle.* Vol. IIId, p. 328. Akademie Verlag, Berlin.

Greif, N. (1967). Cutaneous safety of fragrance materials as measured by the maximization test. *Am. Perfumer Cosmet.* **82** (June), 54.

Handbook of Organic Industrial Solvents (1961). 2nd ed. Technical Guide no. 6. National Association of Mutual Casualty Co., Chicago, Ill.

Jenner, P. M., Hagan, E. C., Taylor, Jean M., Cook, E. L. & Fitzhugh, O. G. (1964). Food flavourings and compounds of related structure. I. Acute oral toxicity. *Fd Cosmet. Toxicol.* **2,** 327.

Joint FAO/WHO Expert Committee on Food Additives (1967). Toxicological Evaluation of Some Flavouring Substances and Non-nutritive Sweetening Agents. *F.A.O. Nutr. Mtg Rep. Ser. no.* 383A, Geneva, p. 12; WHO/Food Add./68.33.

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

Lehmann, K. B. & Flury, F. (1943). *Toxicology and Hygiene of Industrial Solvents.* Williams and Wilkins, Baltimore.

Moreno, O. M. (1972). Report to RIFM, 1 November.

von Oettingen, W. F. (1960). The aliphatic acids and their esters: Toxicity and potential dangers. The saturated monobasic aliphatic acids and their esters. *A.M.A. Archs ind. Hlth* **21,** 28.

Williams, R. T. (1959). *Detoxication Mechanisms. The Metabolism and Detoxication of Drugs, Toxic Substances and Other Organic Compounds.* 2nd ed. p. 319. Chapman & Hall Ltd., London.

Fd Cosmet. Toxicol. Vol. 11, pp. 877–884. Pergamon Press 1973. Printed in Great Britain

Review Section

Safety Regulation in the Real World*

PETER BARTON HUTT

Assistant General Counsel for Food and Drugs, Department of Health, Education and Welfare, Washington, D.C. 20204, USA

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Regulation of the safety of food and drugs should be an extremely simple and perfunctory task. After all, one need only hold up the indisputable scientific facts and compare them with equally explicit statutory requirements. The regulatory result will then ineluctably follow, with the full concurrence and acclaim of the Congress, the regulated industry, the consumer advocates, the academic community and the public at large.

Unfortunately, however, this does not occur in the real world. In the 20 months that I have held my current position, I cannot recall one major safety decision by the Food and Drug Administration—regardless which way it was resolved—that has failed to provoke prolonged, and at times bitter, public dispute. Moreover, even if we had reached exactly the opposite conclusion on any of those decisions, it is unlikely that there would have been any greater or lesser amount of dispute.

In short, public policy design and execution with respect to the safety of food and drugs is highly, and perhaps irretrievably, controversial. It raises a welter of subjective and emotional views that often obscure rational analysis and that can severely hinder regulation by scientific decision-making.

My remarks here will initially outline the statutory mandate for safe food and drugs. I shall then discuss what I discern to be the principal obstacles to decision-making on safety matters. Finally, I shall describe the action that is being taken to improve the situation.

I

Any discussion of decision-making on the safety of food and drugs must begin with an understanding of the broad statutory mandate Congress has given to the FDA.

As early as the Food and Drugs Act of 1906, Congress demanded protection of the public from unsafe food and drugs. That law prohibited the use in food of "any added poisonous or other added deleterious ingredient which may render such article injurious to health". It similarly prohibited the use in drugs of any "ingredient deleterious or detrimental to health".

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When Congress modernized this law in 1938, it was unable to improve upon those general statutory admonitions. The Federal Food, Drug, and Cosmetic Act states that a food may not contain any "poisonous or deleterious substance which may render it injurious to health", and that a drug may not be recommended for any use for which it is "dangerous to health". Even the Food Additives Amendments of 1958 and the Drug Amendments of 1962, which were enacted specifically to provide more stringent protection against unsafe food and drugs, are again couched in very broad and general terms. The food additive provisions of the law require the Food and Drug Administration to consider "safety factors which in the opinion of experts qualified by scientific training and experience to evaluate the safety of food additives are generally recognized as appropriate for the use of animal experimentation data". The new drug provisions of the law state that safety must be shown by "adequate tests by all methods reasonably applicable to show whether or not such drug is safe for use".

The only detailed safety criteria contained in the statute are contained in the famous anti-cancer clauses. In contrast to the public attention they have received, however, those clauses are actually an issue in very few safety decisions made by the FDA—certainly, far less than one-tenth of 1% of those decisions. And in most of the instances where they have been an issue, the matter has been resolved using sound scientific judgement, based upon general principles of food safety, before it was necessary even to consider invoking them. As a practical matter, therefore, the anti-cancer clauses are a relatively insignificant factor in the daily administration of the safety provisions of the law, although they unquestionably present a fascinating subject for discussion at a forum such as this.

In giving the FDA sweeping authority to require that all food and drugs be safe, therefore, Congress has relied upon a very broad and general mandate rather than upon narrow and specific rules. Not one of the critical statutory terms or phrases that control the numerous safety decisions made daily by the FDA is defined in the Act. Nor, indeed, do I believe that they can or should be defined at this point in time. Safety evaluation is today an imprecise and uncertain task. Until the ambiguities and imponderables that now inhere in that task are clarified and elucidated, rigid safety definitions seem unlikely to lead to sound public policy.

Perhaps the best example of the thicket into which any legislative body wanders in attempting to enact any rigid safety criteria may be found in the various anti-cancer clauses now contained in the Act. Regardless of whether one supports or opposes inclusion of a specific anti-cancer provision in the law—and I take no position whatever on that issue—I think we can all agree that the present versions are indefensible from any viewpoint.

In 1958, when it first included an anti-cancer clause in the law, Congress applied it only to food ingredients that were not generally recognized as safe or were not previously approved for use in food by the FDA or the United States Department of Agriculture. From this, one might deduce that Congress favoured the use of old and familiar carcinogens over newly discovered ones.

In 1960, a second anti-cancer clause was enacted as part of the Color Additive Amendments. This time Congress applied the clause to colourings that are permanently approved for use in food and drugs after adequate safety testing, but not to colourings that are only temporarily allowed for such use because of a lack of adequate safety testing. From this, one might deduce that Congress thought that the American public was entitled to at least a few more colourful years of some of its favourite carcinogens before it must face the prospect of a supply of drab food and drugs.

Then in 1962, faced with the inconsistency of its distinction between pre-1958 and post-1958 carcinogens, Congress amended the two clauses to solve the problem. The principal issue was diethylstilboestrol, which had been approved for use by some manufacturers as an animal growth promotant prior to 1958. Those manufacturers who had a pre-1958 approval could make it, and all others could not. Instead of eliminating this distinction, however, Congress added yet another in order to require the FDA to allow production of diethylstilboestrol by all manufacturers. Under the Drug Amendments of 1962—and under a third anti-cancer clause added to the law in the Animal Drug Amendments of 1968—the FDA must now approve a known carcinogen for use in food-producing animals as long as available analytical methodology is unable to detect that carcinogen in the food obtained from the animal. From this, one might deduce that Congress simply wanted to inject a little excitement into an otherwise rather stodgy law, and reverted to that favourite old childhood game of hide-and-go-seek.

Nor is that the last chapter. In 1972, when scientists finally did find diethylstilboestrol in animal livers using radioactive tracers, and the FDA banned it from animal feed with a 5-month phase-out period, the Senate promptly passed a bill to ban its use immediately. The House did not act on that bill. From this, one might deduce that half of Congress sincerely regretted that it had ever wandered into this thicket, and the other half was simply immobilized by the total incongruity of the matter.

This rather incredible chronicle of legislative groping for political and scientific truth holds important lessons for all of us. Congress, in attempting to deal in detail with just one of many thousands of safety issues, has clearly floundered. The rule it has erected is so riddled with exemptions and exceptions as to make it indefensible in its present form. At present, it serves more as a barrier to the removal of unsafe animal drugs from the market than as a measure for public protection. Indeed, that is the very reason why it is so seldom invoked. Its underlying purpose is, and properly should be served by, the general safety provisions of the law.

In the last analysis, regulation of the safety of food and drugs must depend upon informed scientific judgement. The scientific uncertainty that exists at this time simply requires that safety determinations be made more on the basis of subjective evaluation than of objective standards. And as long as this remains the situation, it must be expected that reasonable men can, and frequently will, differ on the judgement made by the FDA in any given situation, and thus on whether the statutory requirement of safety is properly being implemented.

II

The FDA's decision-making process on safety issues, and the public perception of it, are hampered by five basic obstacles. No single one of these obstacles is critical, but their combined impact can at times be severe. And while each of these obstacles may be present to a greater or lesser degree in any particular safety decision, there is probably no major safety issue involving the FDA to which all do not contribute.

First, the scientific-data base is seldom adequate to make a definitive safety judgement on any food or drug.

With every passing year, scientists develop new, more sophisticated safety-testing methodology. Just one adverse finding, from whatever test method employed, seems sufficient today to call into public question the safety of virtually any ingredient used in food or drugs. If the product has been on the market for many years, as is true with much of our

food supply, it is unlikely that it would have been subjected to many, if indeed any, of the scientific tests that are considered commonplace today. It simply is not feasible every year to go back and retest, using the newest methods, all the components of food and drugs that have previously been placed on the market. And even with the most recent additions to the marketplace, it is doubtful whether any substance has been, or can be, so thoroughly tested as to preclude further scientific question.

It has long been recognized that no amount of human or animal testing can ever demonstrate the absolute absence of harm. All that one can ever show with certainty is the existence of harm. The marketing of any product therefore carries with it an inescapable but indeterminable risk. With the recent reports of vaginal cancer in the female offspring of mothers who had used diethylstilboestrol during pregnancy, moreover, this point has become a matter of immediate and serious policy concern to the FDA. At present we have no way whatever of predicting this type of future harm for products about to be marketed, and our ability to monitor the safety of already-marketed products is limited. Even centuries of use of natural substances in the diet, without noticeable adverse effects, cannot be regarded as proof of safety, since it is based only upon uncontrolled observations.

Thus, proof of complete safety appears at this moment to be an illusory goal. Both those who challenge and those who defend the safety of any particular substance can do so with the assurance that information adequate either to support or to refute their contentions is not now available, and may never be. And today's decisions on the safety of food and drugs will therefore inevitably be made on the basis of incomplete scientific information.

Second, even when substantial safety data are available on a particular substance, there is seldom scientific agreement on the meaning or significance of that information.

Scientists have been far more successful in inventing new methods of safety testing than they have been in determining the significance of the results obtained. This is particularly true with the still-evolving animal tests for carcinogenicity, teratogenicity and mutagenicity. The meaning of adverse results obtained from these experiments, and especially their relevance to human use, is usually open to severe scientific disagreement. And of course the likelihood of obtaining at least one adverse or questionable finding increases with every test that is conducted.

Even those animal tests that have become widely accepted by scientists frequently produce results that are variable and inconclusive. Every scientist knows that quite different results can be obtained from a standard test protocol using different animal species, different strains of the same species, different animal rations, different routes of administration and a host of far more subtle variables. Different laboratories not infrequently obtain diverse results even trying to replicate identical testing procedures.

In short, the significance of much of the animal safety testing conducted today is poorly understood, and the widely variable results obtained are subject to differing interpretations. Its usefulness in the design and execution of sound public policy under these circumstances is unfortunately limited. As a matter of practical necessity, therefore, we often regulate more out of fear of the unknown than out of respect and appreciation of the known. And until science begins to bring greater understanding to safety testing, regulation of the safety of food and drugs must be accomplished in the midst of unresolvable scientific disagreement.

Third, even assuming that an adequate basis of scientific data were available, together with scientific agreement on the meaning and significance of the data, there appears to be no public or scientific consensus today on the risk or uncertainty acceptable to justify the marketing of any substance as a food or drug.

To some, who favour a return to more simple days, no risk or uncertainty whatever is justified for any addition of a chemical to food. Such people would, indeed, require a showing of some greater benefit to society before any ingredient is permitted. To others, who see enormous progress in food technology and nutrition from the use of food additives, the usual risks associated with technological innovation are regarded as entirely reasonable. Even in the area of therapeutic drugs, there is intense public dispute about whether, for example, the risks of an abortion outweigh the risks that are raised by the use of diethylstilboestrol as a post-coital contraceptive.

We must recognize that this type of issue presents fundamental differences in philosophical principles, not simply a narrow dispute on technical details. It raises the most basic questions of personal beliefs and human values—the degree of risk or uncertainty that any individual is willing to accept in his daily life. Attempts to resolve it on the basis of rigorous scientific testing or analytical discourse therefore simply miss the point. A mathematical benefit/risk formula or computer programme may eventually be able to quantitate the risk or uncertainty that inheres in a given product, but it is not even relevant to the moral and ethical issues involved in deciding whether that risk or uncertainty is acceptable.

This problem arises whenever new doubts or suspicions are cast upon the safety of an already-marketed substance. Those who favour a very low public risk demand that the product immediately be removed from the market. Those who advocate a higher risk demand that it remain on the market until it is shown to be unsafe. If, as I suspect will happen, we eventually prove that many of our basic foods and drugs contain at least trace amounts of highly toxic substances—including carcinogens, teratogens and mutagens—the public will simply have to face these issues in a more forthright way than it has up to now.

One does not need a degree in science to hold and express deeply-felt beliefs on the degree of risk or uncertainty that society should accept from food and drugs. Nor, indeed, does a scientific background equip one with any greater insight into the intricacies of this type of policy issue or any more impressive credentials or greater authority to act as an arbiter in resolving these matters. As long as we remain a free society, these basic philosophical principles will, and properly should, remain the subject of intense public scrutiny and debate.

Fourth, there is enormous and continuing public pressure for the FDA to resolve whatever may be the latest current safety issue promptly and decisively.

Delay and indecision weaken public confidence and intensify fear and concern. Industrial representatives, faced with potential harm to their economic interests, demand reassurance that the public need fear no danger. Consumer activists, sensing a further victory in their war against unsafe products, intensify the public campaign to discredit the suspect product. Congressmen, reacting to the legitimate concern of their constituencies, demand a prompt resolution. The media, recognizing a story of interest to the entire public, do not fail to give it ample prominence. Thus, regardless of the uncertainties and imponderables, a decision must frequently be reached immediately, on the basis of whatever meagre information may exist.

On economic issues, a government agency may be able to take its time, to sift the facts, to make further investigations and to act calmly and deliberately. Certainly, scientists in the academic world have ample opportunity to conduct further studies, obtain additional information and engage in reflective thought, before making difficult judgements on complex scientific issues. In the emotion-charged atmosphere of a botulism or cancer scare, however, that process is necessarily foreshortened. It is simply unrealistic to believe that the FDA can ignore or even long resist the need to act promptly and decisively under those circumstances.

Fifth, regardless of the outcome of the decision, those who disagree with it will continue to pursue the matter through all available channels, while those who agree with it will inevitably remain silent, preparing themselves for the next issue.

Graceful acceptance, or even grudging acquiescence, by those who have lost any important safety decision is a rare exception. And praise or even mild support from those who have prevailed is equally rare.

The price of virtually any major safety decision is at least one Congressional hearing, and perhaps more—regardless which way the decision goes. At least one Congressman will be persuaded, sooner or later, that important facts were not adequately considered, or appropriate weight was not given to particular viewpoints, and therefore that the entire matter should be subjected to further public scrutiny. This is obviously an important and appropriate Congressional function.

For those in industry or consumer organizations whose views were not accepted by the Agency, moreover, there is ready access to the courts. This is not only their right, but indeed their duty when they believe we have made an incorrect decision or acted unlawfully. Quite frequently, the economic stakes are extremely high. I have framed a law that, while not immutable, certainly has general application today: industry is likely to challenge in the courts any FDA action where the net adverse economic impact exceeds the legal fees involved.

Thus, no matter how promptly and decisively the matter is resolved, it usually does not end there. It continues to reverberate in the media, in Congress, in the courts and in public debate, for months or years to come. Invariably, new scientific evidence will come to light on which one side of the issue or the other will find new sustenance. It is not at all surprising that the 1959 cranberry episode, the 1962 thalidomide tragedy and the 1968 cyclamate ban are still discussed as though they happened yesterday. And I am confident that our current ban on diethylstilboestrol as an animal growth promotant, and whatever decision is ultimately reached on saccharin, will still be debated in lively terms 20 years hence. Nor is there any greater likelihood that a scientific or policy consensus on these issues will ultimately be reached then than there is that one could be reached at this moment.

As a lawyer, I am not only accustomed to the adversary process, but also a strong advocate of it. Nevertheless, we must be careful to prevent trial by combat replacing reasoned decision-making on important safety issues.

III

In the midst of all this disarray and confusion stands the FDA, bearing its heavy statutory responsibility of assuring the safety of all food and drugs marketed in this country. With inadequate scientific data, with fundamental scientific disagreement on technical issues and public disagreement on policy issues, with the necessity to act decisively and promptly and with the assurance of widespread dispute about whatever action emerges, the Agency daily makes some of the most important public policy decisions that directly affect all of our lives. I say this with neither exaggeration nor rancour, but simply with candour, based upon the insight gained from active participation in this process. Nor do I believe that it is reasonable to expect that these very real obstacles will change dramatically, much less disappear, in the near future.

These obstacles have clearly taken their toll. Public and Congressional confidence in the ability of the FDA to carry out its statutory responsibilities has unquestionably been

undermined. It has thus become apparent that the Agency must meet this challenge or face potential destruction.

Instead of throwing up our hands in despair, however, we have already instituted major changes in the decision-making processes of the FDA to accommodate and even assimilate these obstacles. Faced with deeper public concern about the safety of food and drugs than ever before, we have begun to open up the Agency's deliberations to substantially greater public scrutiny and participation, and thus equally greater public accountability, than perhaps any government agency in history. These changes, which are still in progress and will undoubtedly not be completed for some time, involve three essential elements.

First, we are developing new procedural mechanisms to guarantee that all interested persons have access to the FDA before important decisions are made. These procedures cannot permit Agency decisions to be tied up forever in needless red tape that only delays the process, but they must provide all segments of the public—consumer activists, the regulated industry, the academic world and the public at large—with information about what the Agency is considering and with a meaningful opportunity for their voices to be heard before a decision is reached.

We have, in the past 18 months, developed such procedures for review of over-the-counter drugs and biological products. Anyone may submit written information or may make oral presentations to the reviewing panel at its frequent meetings. Our new procedures, which for the first time permit the FDA to impose additional safety testing for already-marketed food ingredients, encourage petitions by any person who wishes to designate specific testing that should be required for a particular substance. We have just announced a public hearing to consider internal guidelines that will govern the formulation and labelling of a class of prescription drugs—the first such hearing in the Agency's history. These are just a few examples of the changes taking place. And we are now beginning to think again about all the procedures by which the FDA promulgates its regulations and makes its decisions in order to assure that this new policy is in fact fully implemented.

Mr. Justice Frankfurter pointed out 30 years ago that "the history of liberty has largely been the history of observance of procedural safeguards". I am not so naïve as to believe that, simply by improving our procedures, a scientific and lay consensus will be reached on difficult safety issues and our critics will be stilled. Greater public access and representation of divergent viewpoints will, however, inevitably bring with it the beneficial impact that results from any person feeling that he has, in fact, participated in the decision-making process.

Second, in addition to guaranteeing more direct and immediate access to the Agency's decision-making, we are broadening the base of many of our decisions. Since subjective judgement plays such a large role in safety decisions, we are attempting to make certain that the most informed and respected judgement the country has to offer on these matters is in fact brought to bear on them. During the past 3 years, the FDA has increasingly relied upon independent technical advisory committees, consisting of scientific experts, to provide advice on major regulatory issues. In the review of over-the-counter drugs and biological products, moreover, we have now gone one step further and have included non-voting consumer and industry members on the technical committees.

It seems clear that, in addition to placing even greater reliance on independent advisory committees in the future, we must begin to include a broader representation of interests on these technical committees, rather than adhering strictly to scientists with specific expertise in the issues immediately before the committee. For as I have already indicated, the difficul-

ties we encounter in our decisions increasingly involve fundamental philosophical principles and basic questions about the quality of life that trouble our entire society, as well as detailed scientific judgements. Deliberation on these issues properly deserves representation from the entire public.

Third, we are more fully articulating our policy decisions and the reasoning behind them. The public cannot be expected to understand and accept decisions that are nowhere explained. Both the rationale for each decision and any underlying documentation must be laid bare to critical scrutiny.

The FDA has publicly committed itself to this goal, with results that have been seen daily in the Federal Register for over a year. The explanation of Agency actions, contained in lengthy preambles to new regulations, frequently takes up five to ten times as much space as the regulations themselves. And the procedures for the current review of OTC drugs, biological products and *in vitro* diagnostic products, provide for the public release of virtually all data provided by industry—including volumes of heretofore unpublished scientific information—upon publication of the proposed regulation.

We have not yet fully solved the problem of making public all of the scientific information that has been accumulating in the FDA files since 1906. That is due to problems of resources and logistics to do the job, however, and not to any desire to retain it as secret. Nor have we fully settled the question of what data and information provided to the FDA by industry represent true trade secrets that deserve to be held by us in strict confidence. We are hopeful that both of these problems can successfully be resolved in the near future.

Opening up FDA deliberations in these three ways has already substantially improved both our ability to handle difficult safety issues and the public's appreciation of what we are doing. We are, in short, beginning to surmount the obstacles that I have described, and I anticipate enormous further progress in this endeavour in the next year.

IV

Thus, I am extremely proud of the FDA's recent achievements, and very optimistic indeed for its future, in spite of the very difficult obstacles it must overcome to achieve full and fair enforcement of the law. The fact remains that, if there were no FDA, one would have to be invented. Some government regulatory agency must be responsible for making the daily decisions as to whether a given food or drug is sufficiently safe to be permitted on the market. The tremendous success of the FDA in carrying out this difficult responsibility is shown by the fact that, in spite of the hundreds of thousands of foods and drugs marketed, the number of known instances of harm that could have been avoided by regulatory action has been extraordinarily small. We intend simply to continue making the most responsible judgements possible, on the basis of the best available information and advice, and we welcome the help of everyone who believes, as we do, in the vital importance of this mission to the public welfare.

Fd Cosmet. Toxicol. Vol. 11, pp. 885–886. Pergamon Press 1973. Printed in Great Britain

REVIEWS OF RECENT PUBLICATIONS

Health Hazards of the Human Environment. World Health Organization, Geneva, 1972. pp. 387. £4.40.

Since its inception in 1947, the World Health Organization has devoted a large share of its resources to the collation and dissemination of information, on the premise that the exchange and distribution of scientific knowledge is mandatory if a universal improvement in public health is to be promoted. The publication of *Health Hazards of the Human Environment* is in keeping with this principle.

The book is intended to serve as a concise handbook for public health authorities and consequently deals with a wide spectrum of topics in a synoptic form. This limitation is countered, however, by the provision of an adequate bibliography at the end of each chapter.

A large proportion of the world's population resides in the developing countries. These countries, in general, do not possess the resources to provide efficient sanitation or adequate supplies of food and water and, as a result, communicable disease and malnutrition pose the greatest threats to human health. Industrialization, 'civilization' and the attendant improvement in public health have greatly reduced the incidence of communicable disease in the more economically advanced countries, but have introduced new and insidious threats in the form of a variety of chemical, physical and social factors.

This WHO publication is arranged in four main sections, each subdivided into chapters. The first part deals with the community environment. The actual and potential hazards involved in man's assocation with the atmosphere, water and food are described, as well as those hazards specifically encountered in the home and at work. The following part, on chemical contaminants and physical hazards, opens with a chapter on laboratory toxicity tests, providing a brief survey of their aims, limitations and significance. The remaining chapters in this part are concerned with selected environmental pollutants and the special problems posed by mutagens, carcinogens and teratogens. Separate chapters are also concerned with the adverse effects of ionizing and non-ionizing radiations and noise.

There is a prime need in the public health field for the development of an integrated system of surveillance and monitoring, to provide an early-warning system for the adverse effects of the environment on human health. WHO has been engaged for some years in the collation and interpretation of data on communicable diseases and adverse drug effects. Part III attempts to indicate those additional areas in which surveillance is most needed and the lines along which it should be organized. The closing section is concerned with the environmental health standards and criteria that have been and still need to be established and also with methods and mechanisms of control procedures. Much consideration is given to problems of basic sanitation and methods for overcoming them, since in this field lies a major means of controlling many communicable diseases.

Some of the topics presented are of a controversial nature but, generally speaking, the book deals objectively with the subject matter. Its content and presentation make it a suitable source of reference not only for those professionally involved in the problems it covers but also for others interested in the changing environment and its interaction with human life.

Fd Cosmet. Toxicol. Vol. 11, pp. 887–894. Pergamon Press 1973. Printed in Great Britain

BOOK REVIEWS

Sorbinsaure. Chemie—Biochemie—Mikrobiologie—Technologie. Band II. Biochemie—Mikrobiologie. By E. Lück. B. Behr's Verlag GmbH, Hamburg, 1972. pp. 127.

We have already reviewed two volumes of this wide-ranging survey on sorbic acid. Volume I dealt principally with the chemistry of the compound and its derivatives (*Cited in F.C.T.* 1971, **9**, 431) and Volume III with its uses and technology (*ibid* 1972, **10**, 234). The project was completed in 1972, with the publication of Volume II, covering the biochemistry and physiological effects of sorbic acid in plants and animals and particularly its activity against micro-organisms.

A brief opening chapter reviews the evidence for the natural occurrence of sorbic acid derivatives in mountain ash berries, aphids and fungi. This is followed by an account of the physiological behaviour of sorbic acid and its salts, esters and other derivatives. The toxicity of sorbic acid has been studied extensively and results of acute, subacute and long-term toxicity studies are discussed in some detail. Its biochemical effects and metabolic fate are also reviewed, as are its actions on the skin, an important aspect in view of its use as a preservative for cosmetics preparations.

About three-quarters of the book, however, is devoted to the capacity of sorbic acid to inhibit the activity of an impressive number of bacteria, algae and fungi. Of prime importance in this connexion is its interference in the functioning of numerous enzyme systems, and particularly those of the citric acid cycle and related reactions. Among the details provided are data on the degree of inhibition of different enzymes achieved by specified concentrations of sorbic acid and, in tables occupying a total of over 40 pages, on the concentrations of the acid that are effective in inhibiting different bacteria and fungi in various media. The influence of other preservatives and antibiotics on the effectiveness of sorbic acid against yeast and some bacteria is also given some attention.

This publication adds another 600 references, including some 1971 papers, to those already collected together from the literature that has accumulated on this preservative. Both newcomers to the subject, faced with the task of familiarizing themselves with the current state of play, and those needing to locate specific items of information will surely find their task considerably eased by the painstaking work that has gone into these three volumes.

Environmental Mercury Contamination. Edited by R. Hartung and B. D. Dinman. Ann Arbor Science Publishers Inc., Ann Arbor, Mich., 1972. pp. ix + 349. £9.40.

Mercury in four parts: occurrence in the environment and in man, methods of analysis, environmental dynamics and biological effects—mention of these sections suffices to indicate the importance of this book to everyone concerned with food, the environment or any

product that may contain mercury in some form as a deliberate or unavoidable ingredient or contaminant.

The contributions under the various headings stem largely from a conference in which the emphasis was obviously on current problems posed by environmental mercury contamination. Such discussions would have been provided with better perspective if data had been included on the long-established presence of mercury in fish (Barber *et al. Science, N.Y.* 1972, **178,** 636; Harris & Karcher, *The Chemist* 1972 (May), 176) and on the natural geological levels of the element in soil, water and air. In the absence of this background information, one gains the impression that mercury is a complete newcomer in water, soil, food chains and air. Undoubtedly part of the problem is of recent origin, and it is for this aspect that remedies must be sought. In our search for understanding and for preventive and corrective measures, the excellent contributions presented in this volume will prove invaluable.

Metallic Contaminants and Human Health. Edited by D. H. K. Lee. Academic Press, New York, 1972. pp. xvii + 241. £3.25.

The short penultimate chapter of this book presents some cases of adverse effects in man resulting from exposure to various metals in different situations and, in doing so, provides sufficient justification for the publication of this volume. Other chapters on individual metals or groups of metals are written by different authors, but the comparable format of these contributions makes for a neat and easily followed text. Eight metals, mercury, lead, cadmium, beryllium, chromium, manganese, nickel and vanadium, are covered, together with arsenic and, perhaps rather oddly, a chapter on fluorides.

Some information is presented on the natural occurrence and technological uses of each of these environmental contaminants, and also on the ways in which human exposure may occur. The bulk of each chapter contains an account of the biological activity of the metal, including clinical observations of its effects in man. A chapter on the nutritional aspect of metals and another on standards and analytical techniques are useful adjuncts to the main text.

The Editor's stated aim was to avoid over-complication and to prevent each chapter from becoming a highly detailed review specifically designed as a source of reference for the expert. In this he has succeeded, but the result is a volume that will be of value for the education of students, but is too brief and non-critical for the expert and much too technical for the layman.

Unlike the many other volumes on metal toxicity published recently, this volume opens with a chapter on the general concepts of environmental toxicology and, as might be expected, this is extremely controversial. It adopts the rather unusual attitude that biological effects need not be classified as harmful or harmless, but rather as wanted or unwanted. Perhaps space restrictions were responsible for the somewhat inadequate coverage of this topic.

The original references quoted provide a satisfactory background for the text if the book is to be used for educational or training purposes. The lack of an index is to some extent overcome by the precise subjects covered in each chapter.

A Guide to Marine Pollution. Seminar in Conjunction with the FAO Technical Conference on Marine Pollution and its Effects on Resources and Fishing. Edited by E. D. Goldberg. Gordon and Breach Science Publishers, New York, 1972. pp. x + 168. £4.15.

Contrary to the impression that some potential readers may gain from the title, this text is limited to an account of the contamination of oceans by man-made chemicals. Moreover, it is not a layman's guide but a reference source of data and methods for those actively engaged in the problems of oceanic ecology. The book is, in fact, an expansion of the Final Report of the Seminar on Methods of Detection, Measurement and Monitoring of Pollutants in the Marine Environment, organized by FAO with the support of UNESCO, IAEA and several other international organizations and held in Rome in December 1970.

The essential usefulness of the text can therefore only be assessed by the research worker in this particular field. Nevertheless, to those involved in toxicology and to others employed by the chemical industry, the types of experimental approach to various problems, the physical and analytical techniques used, the schedules of monitoring envisaged and the recommendations for further work will be of much interest. In particular, the facts and ideas expressed may assist in bringing together the various existing factions, a necessary event if the causes, nature and size of the problem of ocean pollution are to be realistically assessed.

Pharmacology and Toxicology of Naturally Occurring Toxins. Edited by H. Rašková. International Encyclopedia of Pharmacology and Therapeutics. Section 71. Vol. II. Pergamon Press, Oxford, 1972. pp. ix + 299. £7.50.

A major part of this volume, one of a large series, is concerned with the pharmacology and toxicology of toxins from some of the 1000 or so poisonous or venomous marine organisms that have been classified. Therapeutic problems associated with this chemically heterogeneous group of toxins are also given extensive treatment.

The venoms of insects and arachnida are given much less attention, although they constitute an enormous variety of toxic substances and produce a wide range of symptoms. The rather thin coverage of this subject is due largely to an incomplete understanding of the mechanism of action of these venoms, some of which act directly on the animal structures while others release autopharmacological substances which are toxic to the victim.

The rest of the volume is concerned with the pharmacological and toxicological properties of mycotoxins isolated from fungal species that contaminate our food. The potential hazards associated with chronic mycotoxin poisoning are manifold, as is evident from the geographical and botanical ubiquity of these fungi and their wide range of chemical properties and biological effects. However, the 'toxic' fungi so far detected are largely confined to those with identifiable acute toxicity. The data on chronic toxicity that do exist are examined in this volume in the context of the wide gap that exists in this area of knowledge. Wide interest in mycotoxicosis was aroused about 30 years ago with the studies on "yellowed rice" in Japan, and the first species considered in this review, *Penicillium citreo-viride* Biourge, was used in an attempt to isolate the yellow toxin. The isolation and properties of this toxic principle are discussed in some detail, following some interesting sidelights on Japanese social history. The author postulates an aetiological connexion between yellowed-rice mycotoxicosis and the condition known as acute cardiac beri-beri, but also considers some of the other factors that may be influential in the development of this disease.

Attention is also given to hepatotoxic fungi present in mouldy rice, notably the species *P. islandicum* Sopp. A methanol extract of the cultured fungus has been shown to produce two independent hepatotoxic effects, associated respectively with a chlorine-containing peptide (cyclochlorotine) and a lipophilic compound (luteoskyrin). The short- and long-term toxicological effects of these active principles are examined in detail at both the morphological and biochemical levels.

Mycotoxins from *Aspergillus flavus* are reviewed in brief, and the author attempts, where possible, to correlate the effects of individual aflatoxins with observed biochemical changes. The volume ends with a summary of the toxicology of mycotoxins isolated from species of Fusaria and from several other types of fungi. This and other sections of the work are completed by an extensive list of useful references.

Overall, the book provides an interesting and readable account of naturally-occurring toxins, but retains the systematic approach necessary in such a complex field. Although the three main groups of toxins are reviewed by different authors, with their individual styles, a degree of continuity is maintained throughout.

The UFAW Handbook on the Care and Management of Laboratory Animals. Edited by UFAW. 4th ed. Churchill Livingstone, Edinburgh, 1972. pp. x + 624. £7.00.

This is the fourth edition of a handbook which is widely accepted as the standard book of reference for all laboratory workers using experimental animals. Its 53 chapters are all written by experts in the various fields of laboratory animal care.

The first quarter of the book deals with the theoretical backbone on which the present-day care and use of laboratory animals is built. It includes chapters on genetics, nutrition, hygiene and gnotobiotics and provides extensive information on subjects ranging from the general principles of designing an animal house to the techniques of painless killing. Scientific and senior technical staff with a good background knowledge of these subjects will find these discussions valuable and will welcome the clarity of presentation.

The rest of the book is concerned with the wide range of animals likely, and not so likely, to be required for laboratory investigation. Information on general biology, husbandry, breeding and diseases is given, and biological data and laboratory procedures are also included for most of the species considered. The chapters on the more common animals overflow with useful facts and references, and those dealing with the rarer species provide enough basic information to encourage a confident approach to their husbandry. The chapter on primates is worthy of special mention. There is a useful general chapter on ungulates and those who require fuller information are referred to a separate publication, *The UFAW Handbook on the Care and Management of Farm Animals* (published by E. & S. Livingstone, Edinburgh, in 1971).

The whole work is illustrated with half-tones and line drawings of a high standard. This much-improved edition of the Handbook should be available in every laboratory where animal experiments are carried out.

Autoradiography for Biologists. Edited by P. B. Gahan. Academic Press, London, 1972. pp. xi + 124. £2.

Autoradiography is something of a hybrid subject. On the practical side there are the

relatively simple techniques of cutting thin sections of biological material and developing photographic emulsions, while on the theoretical side there are the inevitable mathematics and physics associated with radioactive decay, crystal size of emulsions and quantitation techniques.

Dr. Gahan has collected together six chapters on autoradiographic technique written by workers experienced in both the theoretical and the practical aspects of the subject. To quote from the preface: "The authors have selected a series of methods of which they have personal experience and which have yielded consistently good results over a number of years. Not all the methods described in the literature are quoted, since this book was not intended to be a review of autoradiographic techniques, but rather a "cook-book"."

The first chapter deals with the theory of autoradiography and a superficial glance at the algebraic equations may be sufficient to discourage the beginner. However, beneath this, and written in simple terms, are a consideration of the principles of radioactive decay, a description of types of photographic emulsion and a section on artefacts. Macro-autoradiography, the subject of the second chapter, is known more commonly, but incorrectly, as whole-body autoradiography. Step-by-step instructions are given on the production of autoradiographs of whole animals. This procedure is of particular importance for demonstrating the *in vivo* distribution of drugs, food additives and pesticides, together with their degradation products. Two chapters deal with the basic techniques of stripping film and liquid-emulsion autoradiography, while the remaining ones are more specialized. The problems associated with studies of diffusible substances are well documented and the final chapter is devoted to high-resolution autoradiography. This contribution is of paramount importance to workers interested not just in the localization of radioactive material at the cellular level but in the quantitative examination of subcellular organelles.

The text is well endowed with diagrams and photographs and the information provided should enable even the beginner to produce good autoradiographs. Altogether, this book is a 'must' for any laboratory engaged in autoradiographic studies.

Transport and Accumulation in Biological Systems. By E. J. Harris. 3rd ed. Butterworths, London, and University Park Press, Baltimore, 1972. pp. vii + 454. £8.50.

The principal author has enlisted the help of several distinguished colleagues in preparing this new edition of a well-established book. The last edition was published 12 years ago, and a glance at the publication dates of the 1300 references cited in the current edition shows how much new information has been included.

Transfer across cellular membranes is the main topic under consideration, and particular attention has been paid to the role of membranes in cell function. Since mechanisms of transfer are dealt with at the molecular level, a reader with only a superficial knowledge of the factors influencing transport and accumulation may find the text somewhat heavy going. On the other hand, much will be gained by anyone with a good understanding of the physico-chemical properties of cells, since different types of membrane are considered individually and in detail. The use of artificial membranes, for example, as well as the use of tumour cells, erythrocytes and toad-bladder epithelial cells as membrane models is described.

For the electrophysiologist there are chapters devoted to ion flow and conduction across nerve and muscle membranes, while for the microbiologist there is a chapter on ion flow across the membranes of yeast and other micro-organisms.

Anyone who has mastered the enzymology of mitochondrial function but who finds it hard to relate theory to the actual events occurring at the subcellular level should read the author's own chapter on this subject. As in the rest of the book, the explanations of the ways in which the intracellular environment is maintained in a favourable state go a long way to making theoretical biochemistry come alive.

Keratins. Their Composition, Structure and Biosynthesis. By R. D. B. Fraser, T. P. MacRae and G. E. Rogers. Charles C. Thomas, Springfield, Ill., 1972. pp. xi + 304. $16.75.

Generally this is a well-written and readable book, which contains much useful and up-to-date information.

After a short introductory chapter, the extraction, purification and properties of solubilized keratins are described, the primary structure of various keratins being discussed here. Consideration of the secondary and tertiary structure of keratins follows a chapter on "Histology and Fine Structure" and is based mainly on evidence from X-ray crystallography. About a third of the book deals with the biogenesis of keratins, including all aspects of their synthesis and further information on the electron microscopy of these materials in the epidermis, hair and other appendages. Control of synthesis is discussed, particularly in relation to the time of synthesis of the various keratin constituents. The book ends with a chapter on the evolutionary aspects of keratin. Nearly 600 references are listed.

The book is concerned principally with mammalian keratin, although some comparative information is given, particularly in relation to feather keratin. Four of the nine chapters are followed by a summary and three by suggestions for further reading. These items are useful and it seems a pity that they were not included in the other chapters. The arrangement of chapters and chapter titles seems a little inappropriate in places. For example, the chapter on histology and fine structure, which deals mainly with the structure of wool fibres, is separated by two chapters on keratin structure from that on 'biogenesis', which provides information on electron-microscopic morphology as well as on keratin synthesis. However, the authors achieve their aim of bringing together the information available on the various aspects of their subject and their book should prove of value both to specialists in this field and to general readers.

Carcinogenesis: Recent Investigations. By F. G. Bock *et al.* MSS Information Corporation, New York, 1972. pp. 204. $15.

Mediators of the Allergic State: Recent Investigations. I. By R. Snyderman *et al.* MSS Information Corporation, New York, 1972. pp. 199. $15.

Adverse Effects of Common Environmental Pollutants. By K. Kay *et al.* MSS Information Corporation, New York, 1972. pp. 240. $15.

The explosion of scientific literature over the last few years is a phenomenon of which scientists are only too well aware, as an ever-increasing proportion of their time has now to be spent in trying to keep abreast of recent developments in their field of research. Consequently one hopes that new contributions to the expanding volume of literature will

have a valid *raison d'être*. On these grounds, it is debatable whether there is much justification for the three publications named above.

The first book cited consists of 17 papers published between 1968 and 1970 and reproduced intact from a dozen research journals. The general theme of the papers is chemical carcinogenesis, but within this wide framework the publications vary from an investigation of melanotic tumours in Drosophila to the carcinogenic effects of urethane in newborn hamsters. No attempt has been made to choose articles covering a particular aspect of chemically induced neoplasia. The book does not present any new or previously unpublished work, or attempt to review the articles selected. Moreover while the papers are perfectly respectable scientifically, they cannot be classified as definitive works in the field of chemical carcinogenesis or as comprehensive articles on a more restricted area of investigation. Altogether it is not easy to decide who is likely to benefit from this compilation.

It is equally difficult to assess for whom the second volume was intended. It consists of a loose collection of 16 papers, nine grouped under the heading "Humoral Factors of Immediate Hypersensitivity" and seven under "Cells and Pharmacological Agents Involved in Immediate Hypersensitivity". All are reprints of papers published during the period 1969–1971 in readily available journals, which any reputable immunologist would consult regularly. The very brief preface claims that "judgement has been made in selecting articles to provide a broad overview of the subject rather than an exhaustive review". This seems to imply that the book is designed for non-immunologists wishing to keep abreast of current research in this field. A few general review articles would have served this purpose far better than this collection of detailed experimental papers dealing with interesting but moderately complex and specialized aspects of immediate hypersensitivity, mostly as seen in experimental animals. The specialist in the field would be well acquainted with the work and require some summary or original conclusion to make the book of any value, while the clinician would require a much broader concept and a more obvious slant towards the practical implications of this experimental work in the treatment of his hypersensitive patients. Finally the general scientist would surely require some introduction to the immunological concept of immediate hypersensitivity to provide a framework for the various papers.

The individual papers are all by reputable immunologists and are excellent in their different ways, but only two of them are "general" papers readily understandable by the non-specialist, and even these deal with only one aspect of a very complex subject. The remaining 14 papers are written by specialists for specialists and require considerable background knowledge if they are to be fully understood and appreciated.

The "common environmental pollutants" considered in the third volume are mainly organophosphorus and organochlorine pesticides. Some consideration is given to the level of these compounds found in human tissues and to the toxic effects of specific pesticides, but such diverse subjects as flying hazards attributable to drugs, alcohol, pesticides and carbon monoxide, both in general aviation and in the aerial spraying of pesticides, and various aspects of the problems of noise and its effects on man have been included. The individual papers, generally adequate in their various ways, have been culled from a wide selection of journals published between 1964 and 1969.

All these three volumes lack any introduction to the subjects covered, contain no general discussion and draw no conclusions. What little preface there is to the individual papers contains needless repetition of information, which might have been avoided by the incorporation of the acknowledgements in the tables of contents. Because of the method of

reproduction used, presentation suffers in all three volumes from a disconcerting variation in type size, style and format. In some articles the print is unacceptably small, and the quality of photographic reproduction is generally poor. It is not at all clear what the publishers intended to accomplish with these books, and in consequence it is difficult to recommend them.

BOOKS RECEIVED FOR REVIEW

Mutagenic Effects of Environmental Contaminants. Fogarty International Center Proceedings no. 10. Edited by H. E. Sutton and Maureen I. Harris. Academic Press, New York, 1972. pp. xiv + 195. £3.50.

Clinical Immunobiology. Vol. 1. Edited by F. H. Bach and R. A. Good. Academic Press, New York, 1972. pp. xiv + 296. £6.

Residue Reviews. Residues of Pesticides and Other Contaminants in the Total Environment. Vol. 44. Edited by F. A. Gunther. Springer-Verlag, Berlin, 1972. pp. vii + 192. DM 52.

Fd Cosmet. Toxicol. Vol. 11, pp. 895–907. Pergamon Press 1973. Printed in Great Britain

Information Section

ARTICLES OF GENERAL INTEREST

ANOTHER INSTALMENT ON ORGANOARSENICALS

The toxicology and metabolic fate of the organoarsenicals (*Cited in F.C.T.* 1966, **4,** 339; *ibid* 1972, **10,** 100) are of importance because of the use of such compounds as therapeutic agents, as growth promoters in pig and poultry feeds and as herbicides. Arsenicals used as herbicides are not an important source of contamination of human food, but they may constitute a major hazard to cattle allowed to graze in areas previously treated with these compounds for agricultural or other purposes.

A recent publication (Dickinson, *Am. J. vet. Res.* 1972, **33,** 1889) has reported an investigation into the toxicity to cattle of monosodium acid methanearsonate (MSMA), which is sometimes used in the precommercial thinning of forests. The study was carried out on a group of seven yearling white-faced cattle weighing initially between 118 and 217 kg. Of the seven, one steer and one heifer were used as controls, two steers and one heifer were given a commercial formulation containing 44·9% MSMA plus other ingredients (principally a dye), and one steer and one heifer were treated with an aqueous solution containing 59% MSMA. The total arsenic content of both preparations was about 21%. The daily dose, administered orally in gelatin capsules, was 10 mg MSMA/kg, and this was continued for up to 10 days.

Two steers died during treatment, the one on the aqueous MSMA solution dying on day 8 and one of the two on the commercial formulation on day 10. Treatment with the commercial formulation was terminated on day 10 and the heifer, which was ill during treatment with this formulation, subsequently recovered. The second steer, however, continued to deteriorate and had to be killed on day 12. The heifer on the aqueous MSMA solution died on day 16, 8 days after cessation of treatment. The two control animals were killed on days 10 and 12.

All the treated animals developed severe diarrhoea and showed a loss of body weight by day 7. Autopsy revealed a haemorrhagic gastritis with mucosal erosion and ulceration and intense hyperaemia. The liver showed foci of coagulative necrosis and there was a marked and widespread renal tubular degeneration. Tissue analysis located the highest concentrations of arsenic residues in the kidney, liver and muscle (58, 27 and 10 ppm, respectively) in the steer that died on day 10. In the steer that survived similar treatment but had to be killed on day 12, however, the amount in the kidney and liver were comparable (26·7 and 27·4 ppm, respectively). Similarly, the arsenic level in the kidney of the steer dying during treatment with the aqueous MSMA solution was 46 ppm while that in the heifer dying some days after cessation of treatment was 3·5 ppm. Yet, while arsenic seemed to be removed relatively rapidly from the kidney, the apparent cause of death was acute kidney damage.

The toxicity of a series of organoarsenicals has been investigated in young turkeys. The compounds concerned (arsanilic acid, carbarsone, nitarsone and roxarsone) are used extensively in poultry diets as an aid in the prevention of blackhead disease or infectious enterohepatitis and/or for improving body-weight gain and feed efficiency. The levels of use recommended for these compounds, however, have been reported to have inconsistent effects on body-weight gain and feed efficiency and an evaluation of their toxicity was considered essential to provide a better idea of the dose that could be tolerated by the birds.

Day-old Large White turkey poults were used in all the feeding experiments. They were divided into groups each consisting of 12 males and 12 females and were fed a standard diet consisting of yellow corn mixed with soya-bean meal, to which was added the particular organoarsenical in the appropriate concentrations. Controls were fed the basic diet alone. Feeding was continued for 28 days and attention was paid to body-weight gain, feed efficiency, long-bone (tibia) ash content and mortality.

Carbarsone (*p*-ureidobenzenearsonic acid) was fed to these poults at dietary levels of 0·03, 0·06, 0·09 and 0·12% (Sullivan & Al-Timimi, *Poult. Sci.* 1972, **51**, 1498). Deaths occurred during treatment only in the females given 0·09 and 0·12% carbarsone (8·3% mortality in each case) and in males given 0·12% (25% mortality). No symptoms of organoarsenical toxicity were detected in this experiment, however, and in a second experiment doses up to 0·16% caused no deaths and the 5·6% mortality (two birds out of 18) associated with a dietary level of 0·32% could have been a chance occurrence. There was no evidence of increased body-weight gain or feed efficiency at any level of treatment, but the data indicated at least a nine- or tenfold safety margin with respect to the recommended level of use of carbarsone in turkey diets (0·025–0·0375%) for the control of blackhead disease.

When nitarsone (4-nitrophenylarsonic acid) was fed to the turkey poults at levels of 0·02, 0·04, 0·06 or 0·08% in the feed, one male but no females died during treatment in each of the two lowest dose groups (and in the control group), approximately half of each sex died by day 28 in the group given 0·06% and mortality reached 100% with the highest dietary level (*idem*, *ibid* 1972, **51**, 1582). In a second experiment using graded dietary levels of nitarsone between 0·01 and 0·05%, the mortality rate increased from 5·6% at the 0·03% level to 11·1% at 0·04% and 55·6% at 0·05%. No advantageous effects on body-weight gain or feed efficiency was observed at any of the dose levels, in fact the body-weight gain was less than that of controls in animals given diets containing 0·03% or more. The signs of nitarsone toxicity were similar to those seen in experiments with arsanilic acid and sodium arsanilate (Al-Timimi & Sullivan, *ibid* 1972, **51**, 111), notably leg weakness, head tremors with closed eyes, some paralysis and loss of appetite, but nitarsone was more toxic than either of these two compounds or carbarsone.

Roxarsone (3-nitro-4-hydroxyphenylarsenic acid) at levels of 0·01, 0·02, 0·03 and 0·04% in the diet also led to a decrease in body weight and an increase in mortality (Sullivan & Al-Timimi, *ibid* 1972, **51**, 1641). This was most pronounced in the group given 0·04%, no males and only 25% of the females surviving at day 28. At the 0·3 and 0·2% levels, the male mortality rates were 50 and 25%, somewhat higher in each case than those in the females. In the second experiment with roxarsone, dietary levels between 0·0025 and 0·3% were fed. There was no significant mortality at the lower levels, but body-weight gain was decreased at 14 days by all levels above 0·0025%. Feed efficiency was improved, however, by all levels up to 0·01% although it was decreased by higher levels. These findings are in agreement with the currently approved levels of use for roxarsone (0·002–0·005% in the diet). The signs of roxarsone toxicity were again similar to those seen with nitarsone.

From this overall study it can be seen that roxarsone was more toxic than the other compounds tested, the 'maximum safe levels' (the highest dietary levels causing no depression in body-weight gain and feed efficiency) established for roxarsone, nitarsone, arsanilic acid, sodium arsanilate and carbarsone, respectively, being 0·005, 0·02, 0·03, 0·04 and 0·32% (*idem, ibid* 1972 **51,** 1641). The minimum levels of the first four compounds shown to depress weight gain were 0·01, 0·03, 0·04 and 0·06%, respectively, while the dietary levels required to cause 50% mortality over 28 days were 0·03, 0·05, 0·08 and 0·10%.

These papers provide a better basis for establishing a safe level for the compounds in respect of turkeys. Fortunately the data do not provide any encouragement for increasing the levels of organoarsenicals in feed. If this had been the case a toxicological reassessment of the residue levels would have been essential.

HYDRAZINE

As is to be expected from compounds of high chemical reactivity, hydrazine and its derivatives initiate a wide spectrum of biological responses in animals and man. Some of these effects are useful therapeutically and others are distinctly harmful. In theory, at least, a large number of electron-deficient regions at the molecular level will be amenable to nucleophilic attack by the hydrazine moiety, assuming that the optimal cellular concentrations and stereochemical conditions are achieved. The rapid rise in the production of these compounds in recent years for use in such diverse applications as high-energy rocket fuels and chemotherapeutics has thus aroused the interest of researchers in a number of biological disciplines, including toxicology.

Carcinogenicity

Hydrazine and some of its derivatives have been shown to be carcinogenic in several animal species—notably in rats, in several strains of mouse and in hamsters (*Cited in F.C.T.* 1971, **9,** 724). Recent studies have tended to centre on the problem of the species- and even strain-specificity of tumour induction by these compounds, and the more ambitious workers have attempted to correlate the structure of various derivatives with effects in specific organs.

Feeding studies already reported have revealed that virtually life-long consumption of 0·012% hydrazine sulphate in the drinking-water initiated lung-tumour growth in inbred C3H and Swiss mice but not in an inbred AKR strain (*ibid* 1969, **7,** 663). From the same source has now come a report that similar administration of an identical concentration of hydrazine sulphate to Syrian golden hamsters had no detectable carcinogenic effect (Toth, *Cancer Res.* 1972, **32,** 804].

Further evidence for the tumour-inducing ability of hydrazine and methylhydrazine sulphates was provided by their administration to Swiss mice from 6 weeks after birth until death at a level of 0·001% in the drinking-water. A significant increase in lung-tumour incidence was reported with both compounds. Similar treatment with 0·01% methylhydrazine enhanced the development of pulmonary tumours by shortening their latent period (*idem, Int. J. Cancer* 1972, **9,** 109).

Following administration of 0·001% 1,2-dimethylhydrazine dihydrochloride (1,2-DMH) to golden hamsters in the drinking-water, the main neoplastic response was in the form of

widespread angiosarcomas of the vascular system, although an appreciable incidence of caecal and hepatic tumours was also evident (*idem, Cancer Res.* 1972, **32,** 804). In females, 89% developed blood vessel tumours and 82% of males were affected. In Swiss mice similarly treated, 98% of females and 92% of males developed these vascular lesions, whereas among controls the incidence was 3% in females and 1% in males (Toth & Wilson, *Am. J. Path.* 1971, **64,** 585). In addition there was a significant increase in the incidence of lung tumours in the mice treated with 1,2-DMH. It is noteworthy that when the compound was administered to hamsters by repeated sc injection in a previous experiment (Osswald & Krüger, *Arzneimittel-Forsch.* 1969, **19,** 1891) no vascular tumours appeared. One possible explanation for this apparent anomaly is that the metabolism of the compound may differ when it is administered by different routes (Toth, *Cancer Res.* 1972, **32,** 804).

The early damage to hepatic vascular tissue that preceded the development of blood-vessel tumours in Swiss mice has been characterized by Toth & Wilson (*Archs Path.* 1972, **93,** 427), using light- and electron-microscope techniques. Changes included vacuolation beneath the vascular basement membranes, together with discontinuity and fragmentation of the membrane structure and sinusoidal dilation. Finally the endothelial cells showed fragmentation and disintegration, damage which was followed by the onset of angiosarcoma although the extent to which these two events were related was not established. No liver-cell, tumours were apparent in this experiment.

It appears, therefore, that symmetrical addition of the dimethyl group into the hydrazine molecule can evoke the production of vascular tumours in both mice and hamsters. This may represent a step in the search for some correlation between the chemical structure of a hydrazine derivative and tumour induction at a specific site, but interpretation of results using various routes of administration and various species presents many difficulties.

1,2-DMH has been reported to produce a high incidence of tumours of the large intestine in rats and mice (*Cited in F.C.T.* 1969, **7,** 662). In an attempt to elucidate the biochemical mechanism underlying this tumorigenesis, ^{14}C-labelled 1,2-DMH was administered sc to mice in a dose of 15 mg/kg, and groups of animals were sacrificed 6 and 24 hours after injection (Hawks *et al. Biochem. Pharmac.* 1972, **21,** 432). Nucleic acids were extracted from the hepatic and colonic tissues, and column chromatography revealed the presence of radiolabelled 7-methylguanine in both fractions. This suggested that 1,2-DMH could act as a methylating agent *in vivo*.

Pegg & Hawks (*Biochem. J.* 1971, **122,** 121) have postulated that 1,2-DMH acts by increasing the activity of the enzymes involved in methylation of nucleic acids. When the hydrazine derivative was administered sc to mice in weekly injections of 10 mg/kg (for males) or 15 mg/kg (for females), intestinal tumours were visible after about 24 weeks. Nodular and normal tissue was removed and extracts were used as a source of methylase activity. Extracts from colonic tumours methylated tRNA from *Escherichia coli* at a much greater rate *in vitro* than did those from sections of normal colon. The relevance of this finding is not clear, however, since elevated tRNA methylase activity may be only part of a general increase in the activation of enzymes involved in the macromolecular syntheses associated with the rapid growth of tumour cells.

Acute and short-term effects

In experimental animals, the most dramatic symptoms of acute poisoning with hydrazine are associated with the central nervous system, high levels leading to characteristic convulsions (*Cited in F.C.T.* 1966, **4,** 193). At lower doses various behavioural effects are seen,

and these have recently been investigated in the rat after ip administration of hydrazine sulphate at seven dosage levels between 1 and 52 mg/kg (Pradhan & Ziecheck, *Toxic. appl. Pharmac.* 1971, **18,** 151). Spontaneous motor activity was significantly depressed at a threshold dose of about 40 mg/kg and hydrazine markedly increased the pause in food-reinforcement behaviour at 13–39 mg/kg. When animals were exposed to a shock-avoidance schedule, 6·5–39 mg hydrazine/kg increased the number of shocks experienced in most cases, although the avoidance response was not dose-related.

Effects on lipid and carbohydrate metabolism

Less dramatic, though equally typical of the effects of hydrazine, are the metabolic responses, which include lipaemia, elevation of free fatty acid (FFA) levels in the blood, depletion of liver glycogen and accumulation of fat in the liver (Clark *et al. Biochem. Pharmac.* 1970, **19,** 1743). Following earlier studies on this aspect (*Cited in F.C.T.* 1966, **4**, 194; *ibid* 1971, **9,** 726), the mechanisms underlying these changes have been investigated by Clark *et al.* (*loc. cit.*) using rats fasted for 48 hours. Administration of hydrazine (1·1 mmol/kg) iv increased serum FFA and reduced liver glycogen, and subsequently triggered a roughly tenfold elevation of hepatic triglyceride levels. These effects undoubtedly reflected the uptake of serum FFA by the liver and its incorporation into triglyceride. In liver, total phospholipid and total cholesteryl ester (CE) showed a similar pattern in rising somewhat above control levels for 24 hours, but total free cholesterol was essentially unchanged. In serum, phospholipid levels increased almost immediately, while CE levels decreased sharply. Minimal CE levels occurred between 12 and 24 hours after hydrazine treatment, their occurrence coinciding with the period of maximal hepatic triglyceride content. It was therefore postulated, in the light of the relatively small increase in serum triglyceride concentration, that the hydrazine interfered with triglyceride secretion by the liver. This effect might have been mediated through an inhibition of phospholipid synthesis, since hydrazine is known to interfere with certain pyridoxal phosphate-requiring decarboxylases, one of which is involved in the biosynthesis of phosphatidyl ethanolamine. A deficiency in the supply of phospholipids would lead to incoordination in the building up of lipoproteins and limit the lipid-secreting capacity of the liver. There are, however, arguments for and against this hypothesis. Also found in this experiment was an early hypoglycaemia, together with a depletion of liver glycogen which failed to return to normal during the 48-hour observation period. These results furnish further evidence of an inhibiting effect on gluconeogenesis, to which we have previously alluded in connexion with the suggestion that hydrazine may inhibit the conversion of oxalacetate to phosphoenolpyruvate (*Cited in F.C.T.* 1971, **9,** 727).

Effects on amino-acid metabolism

It is now well-established that the inhibitory effect of hydrazine on vitamin B_6-requiring enzymes is due to the rapid binding of the intoxicant to the coenzyme, pyridoxal phosphate. Recent attention has been focused on the inhibition of one such group of enzymes, the transaminases, which play an important role in both carbohydrate and protein metabolism. In particular, the activity of glutamic–oxalacetic transaminase (GOT) in the rat liver after injection of hydrazine has been the subject of two recent papers.

In the first study (Lee, *Beitr. Path.* 1970, **141,** 99) a single sublethal dose of hydrazine sulphate (260 mg/kg) was injected into the peritoneal cavity of the rat, and at intervals

between 2 and 24 hours rats were killed and sections of liver were prepared for electron-microscopic examination of mitochondrial GOT activity. Inhibition of GOT activity in the mitochondrial cristae was evident within 4–6 hours, although activity in the mitochondrial surface membranes was apparently unaffected. Inhibition was accompanied by mitochondrial swelling, and both changes were reversible, returning to normal within about 24 hours. The value of this study lies in its indication of a correlation between the biochemical changes induced by hydrazine and functional alterations within the mitochondrion.

The second study (Stein *et al. Toxic. appl. Pharmac.* 1971, **18,** 274) showed the main inhibitory effect of hydrazine hydrochloride to be on extramitochondrial GOT activity, an iv injection of 0·1 mmol/kg being required to reduce this enzyme activity by 40% about 1 hour after administration. A similar degree of inhibition of intramitochondrial GOT activity required either a much higher dose or a longer latent period after a 1 mmol/kg dose of hydrazine. Similar inhibitory responses were achieved *in vitro*. Pyridoxal phosphate or pyridoxamine phosphate partially reversed the inhibitory effects on GOT when added to assay mixtures of fractions from rats treated *in vivo* with hydrazine, but such reversals could not be demonstrated by *in vivo* administration of the B_6 compounds. Such effects on key transamination steps may be responsible, at least in part, for the characteristic hydrazine-induced depression of gluconeogenesis, and may also help to explain the disturbances in hepatic protein and amino-acid metabolism induced by hydrazine in experimental animals.

The effects of hydrazine on the amino acids constituting the urea cycle, and on the enzymes catalysing the corresponding transamination steps, have been investigated by a group of Canadian workers (Roberge *et al. Biochem. Pharmac.* 1971, **20,** 2231). They found that addition of varying concentrations of hydrazine to rat-liver homogenates resulted in a gradual inhibition of citrulline and urea synthesis. Daily ip injections of 32 mg hydrazine/kg (1 mmol/kg/day) for 4 days increased citrulline and urea levels in all the tissues examined (blood, liver, brain and kidney) but did not affect arginine levels. An enhancement of arginosuccinase activity was the only detectable change in the pattern of urea-cycle enzymes after hydrazine treatment. Ornithine ketoacid transaminase was markedly inhibited, however, and probably accounted for the observed accumulation of ornithine. It is known that a high cellular level of this amino acid together with an increase in ammonia production stimulates urea synthesis, of which the rate-limiting step is the condensation of citrulline with aspartic acid; on this basis the accumulation of citrulline is readily explicable.

Percutaneous absorption

As well as being a primary irritant and a potent contact sensitizer, hydrazine may be absorbed percutaneously. In a recent study (Smith & Clark, *Toxic. appl. Pharmac.* 1972, **21,** 186), hydrazine applied to the chests of mongrel dogs in doses between 3 and 15 mmol/kg induced temporary discoloration of the skin. In addition, the amount of sodium pentobarbitone required to maintain anaesthesia was increased, and absorbed hydrazine caused convulsions in three out of 25 anaesthetized dogs. Ten of the 25 dogs died during the first 6 hours after treatment. Plasma hydrazine levels rose over the first 40 minutes and then declined, and urinary excretion of the compound tended to increase with the dose. However, the 15 mmol/kg dose resulted in a relatively high hydrazine concentration in the plasma and this remained elevated throughout the 6-hour observation period, without any comparable increase in urinary excretion. This suggested that the higher dose had damaged

the mechanism by which the compound is normally eliminated from the body. Plasma-glucose levels showed an initial increase, but after about 6 hours the hypoglycaemic state described in other studies was attained in most survivors.

Human exposure

In man, the main health hazards stem either from skin contact with hydrazine or from inhalation of the compound for prolonged periods. In practice, the widespread use of hydrazine seems to have given rise to relatively few reports of toxic effects, but severe possibilities are suggested by a recent report of a worker who died after handling hydrazine once weekly over a 6-month period (Sotaniemi *et al. Annls clin. Res.* 1971, **3**, 30). Each time he handled hydrazine he experienced nausea, tremor and conjunctivitis. Autopsy revealed pneumonia, tubular necrosis and nephritis, together with some hepatic damage. The lesions were similar to those produced by hydrazine in laboratory animals. Although the route of intoxication was not clearly established, it is noteworthy that the hydrazine concentration in the factory air to which the worker was exposed was 0·07 mg/m^3, far below the official limit of 1 ppm (1·3 mg/m^3) recommended for hydrazine in workroom atmospheres in the UK and USA. On the other hand the degree of skin contact was apparently unknown.

Clearly, our extensive knowledge of the effects of hydrazine on experimental animals must be supplemented by epidemiological studies in man, before a sound assessment of the hazards resulting from human exposure to the compound can be made.

CARBON TETRACHLORIDE IN DEFENCE AND ATTACK

The phenomenon of tolerance is a well-known response to the repeated exposure of living organisms to many different situations of stress. It is of particular interest to the toxicologist from the point of view of the protective mechanisms that are called into play after the body is exposed to highly reactive chemicals such as carbon tetrachloride (CCl_4). This compound, for example, not only increases resistance to complex substances such as phalloidin—a toxic principle from the white mushroom, *Amanita phalloides*—but also induces a marked resistance to its own lethal effects. Attention has recently been focused on the mechanism underlying this action in rodents, as may be seen from recent papers from three different laboratories.

In the earliest of these studies (Dambrauskas & Cornish, *Toxic. appl. Pharmac.* 1970, **17**, 83), rats were exposed to levels of CCl_4 vapour ranging from 25 to 6000 ppm for 6 hours/day for 1–5 days, or alternatively were given an oral dose of 3·25 g/kg body weight, and were subsequently re-exposed to a normally lethal concentration of 7500 ppm CCl_4 at various intervals between 24 and 120 hours after pretreatment. Response to CCl_4 exposure was assessed in terms of mortality, blood-clotting time and a number of biochemical studies on serum and tissues.

Animals exposed to 4000 ppm CCl_4 for 6 hours or given an oral dose of 3·25 g CCl_4/kg developed tolerance to normally lethal doses of the same compound. Thus exposure to 7500 ppm CCl_4 for 5·5 hours killed 90–95% of untreated rats, but all rats pretreated by either route at least 48 hours before the second exposure survived the otherwise lethal dose. When the second exposure followed only 24 hours after the pretreatment, 30% of the rats died. A similar degree of tolerance (20% mortality) was seen when rats were exposed to the normally lethal concentration 48 hours after pretreatment with 2000 ppm CCl_4. No

tolerance resulted from pre-exposure to levels below 2000 ppm even when this was repeated on five consecutive days. The significantly high concentration of serum bilirubin and the greatly extended blood-clotting time evident after a single treatment with a high dose of CCl_4 were not observed after similar exposure of the pretreated animals. In addition, serum triglyceride levels were appreciably depressed in unprotected rats but were normal in the protected ones. Liver triglycerides were elevated in both groups, although to a less marked extent in the protected animals. The conversion of CCl_4 to chloroform both *in vivo* and *in vitro*, using livers from normal rats and from rats pretreated with 4000 ppm CCl_4 48 hours before sacrifice, was slower in protected than in unprotected animals, indicating that the development of tolerance was associated with a diminished capacity to metabolize CCl_4. This lends support to the theory that the toxicity of CCl_4 is related to its biodegradation, and possibly to the formation of free-radical intermediates with a greater toxic potential than the parent compound.

More recently, the mechanism underlying this protective phenomenon has been examined at the biochemical level (Glende, *Biochem. Pharmac.* 1972, **21,** 1697). A small (0·4 g/kg) non-lethal dose of CCl_4 administered to rats by stomach-tube was remarkably effective in protecting the animals against a subsequent normally-lethal dose (6–7·5 g/kg) of the solvent. A 24-hour period was required for the rats to develop complete resistance to a dose equivalent to the LD_{95} and protection was found to last for about 3 days. Immediately after administration of the initial dose of CCl_4, liver-microsomal aminopyrine demethylase levels and the detectable level of cytochrome P-450 began to fall, reaching about 25% of the control levels after 24 hours. The depression lasted for about 4 days, and was followed by a period of slow recovery. The observed parallel between depressed microsomal mixed-function oxidase activity and resistance to CCl_4 lethality again supports the conclusion that metabolism of CCl_4 is an important factor in its toxicity. More specifically, it appears that the toxic effect is related to the action of the liver-microsomal drug-metabolizing system on CCl_4.

The third report (Gerhard *et al. Virchows Arch. Abt. B Zellpath.* 1972, **10,** 184) is aimed at correlating the biochemical and morphological effects evoked in a CCl_4-damaged mouse liver by a second dose of CCl_4. After a single ip dose of 1 ml CCl_4/kg (approximately 1·6 g/kg), 98% of the animals survived but there was complete destruction of all the CCl_4-sensitive hepatocytes, located in the centrilobular area and accounting for more than half of the liver volume. Over a 10-day regeneration period, the liver parenchymal cells were completely resistant to any further damage by CCl_4, even at five times the normally lethal dose. After regeneration, the histological picture was again normal and CCl_4-sensitivity was restored. Cell damage due to a second dose of CCl_4 given 12–15 days after the first was again confined to the centrilobular area, and both the time-course and the extent of damage mirrored those after a single toxic dose of CCl_4.

Continuous infusion of [^{3}H]thymidine led, some 4 days after a single CCl_4 injection, to 100% labelling of the peripheral liver cells, the cells undamaged by the administered CCl_4, and thus demonstrated the occurrence of regeneration to replace the dead cells. Only a proportion of such labelled cells participate in the mitotic replacement of necrotic cells, however; the majority of cells with a high tritium uptake do not divide but remain in a polyploid state. Nevertheless, it appears that the regenerated liver is composed of an entirely different population of cells formed either by mitosis or by polyploidy. Subsequently the cells of the centrilobular region regain their sensitivity to CCl_4.

PURSUING THE THREAD OF OCHRATOXIN

The ochratoxins are a group of isocoumarin derivatives produced as metabolites by several food-spoiling fungi, including *Aspergillus ochraceus*, *A. melleus*, *A. sulphurea* and *Penicillium viridicatum*. Ochratoxin A (OA), 7-carboxy-5-chloro-8-hydroxy-3,4-dihydro-3*R*-methylisocoumarin linked by its 7-carboxyl group to L-β-phenylalanine, produces hepatic and renal damage in some animal species and possibly in man and is well known as a metabolite of *A. ochraceus* (*Cited in F.C.T.* 1972, **10,** 276). A recent study (Ciegler *et al. Naturwissenschaften* 1972, **59,** 365) has also demonstrated the ability of various species of Penicillium to synthesize this toxin. Cultures were obtained from mould-fermented sausages and grown at 25°C for 10–12 days in YES medium. Thin-layer chromatography of extracts of the various culture media showed that 17 out of 422 cultures incubated, representing three or perhaps four distinct species of Penicillium, were capable of OA production, in addition to *P. viridicatum*, already known to synthesize this toxin. None of these 17 isolates produced OA on infected sausages in up to 70 days of ripening, however.

Almost equally toxic are the methyl and ethyl esters constituting ochratoxin C (OC), but the deschloro compound, ochratoxin B (OB), appears to have much less effect in test animals (*Cited in F.C.T.* 1972, **10,** 276). In a study reported by Chu *et al.* (*Life Sci. Part II*, 1972, **11,** 503) the role of the phenolic hydroxyl group in the dihydroisocoumarin ring in determining ochratoxin toxicity in day-old chicks has been demonstrated. Acute toxicity was found to correlate directly with the acid dissociation constant of the phenolic group. Thus, OA and OC were the most toxic (LD_{50} 135–166 and 216 μg/chick respectively) and OB was much less toxic (LD_{50} 1900 μg/chick), while *O*-methylated OC and the acid hydrolysis product of OA (ochratoxin α; Oα) caused no mortality in doses of 500 and 1000 μg/chick respectively. A phenolic group in its dissociated form thus appears to be necessary for ochratoxin toxicity, a concept in keeping with the known interaction between phenolic groups and body proteins and enzymes. In another paper, Chu & Chang (*J. Ass. off. analyt. Chem.* 1971, **54,** 1032) confirmed that OA and OC had LD_{50}s in day-old chicks of 166 and 216 μg/chick, respectively, and that hydrolysis of OA effectively detoxicated the ochratoxin.

The binding of ochratoxins to body proteins and other macromolecules is obviously important in determining body transport of the toxins. Chu (*Archs Biochem. Biophys.* 1971, **147,** 359) has demonstrated by Sephadex-gel filtration and equilibrium dialysis that 1 mol bovine serum albumin (BSA) can bind 1·87, 2·23 and 2·47 mol OA with binding constants of $3{\cdot}17 \times 10^5$/mol and 1·86 and $3{\cdot}17 \times 10^6$/mol at 25, 12 and 6°C respectively. No binding was observed between OA and α-globulin. Spectrophotometric and spectrophotofluorometric examination of the OA-BSA complex demonstrated maximum complex formation at pH 7–10·5, with possible dissociation above pH 10·5. These findings suggest that serum albumin is capable of serving as a transport agent for ochratoxins, allowing constant liberation of the toxin from the complex to the target tissues.

Excretion characteristics of OA have been the subject of a study by van Walbeek *et al.* (*Toxic. appl. Pharmac.* 1971, **20,** 439) in which rats were given daily intragastric doses of 500 μg OA or else an average of 250 μg ochratoxin daily in a barley culture of a toxigenic strain of *P. viridicatum* for 6 days. The daily excretion of OA in urine and faeces was slightly more than 10% of the intubated dose, part of the toxin being hydrolysed to Oα. Both forms of treatment induced lesions of the kidney tubules, and on day 5 of the experiment the liver and kidneys of animals given the mouldy-barley culture contained small

accumulations of OA, but not of Oα. In the intubated animals there was little toxin accumulation in the liver and kidneys.

The known toxic effects of ochratoxins include fatty infiltration of the liver (*Cited in F.C.T.* 1967, **5,** 730) with glycogen accumulation (*ibid* 1969, **7,** 401). In cockerels, they have been associated with nephrosis, hepatic degeneration, enteritis and interference with bone-marrow function (*ibid* 1972, **10,** 276). In an experiment in which hens were fed from the age of 14 weeks to 1 year on diets containing 1, 2 or 4 ppm ochratoxin (Choudhury *et al. Poult. Sci.* 1971, **50,** 1855), liver and kidney lesions together with severe emaciation and a high incidence of morbidity and mortality were seen during the first 6 weeks in birds on the higher dose levels. Delayed sexual maturity and reduced egg production was apparent in all the treated groups. The impairment in egg production was clearly dose-related and most survivors of the group given the 4 ppm feed failed to lay at all during the year. Hatchability and the growth of the resulting chicks were impaired in the other groups, but the carry-over effect of ochratoxin on chick growth had faded by the end of week 4. This demonstration of the interference of ochratoxin in the reproductive processes of chicks brings to mind again the suspicion that this toxin may be a factor in some outbreaks of bovine abortion (*Cited in F.C.T.* 1972, **10,** 606).

One of the Penicillium species thought to be a likely source of ochratoxin was *P. ochraceum*. The toxicity of several isolates of this species has been demonstrated by Carlton *et al.* (*Toxic. appl. Pharmac.* 1972, **21,** 130), who fed mice on diets containing rice cultures or fungal mats of such isolates. Except for a reduction in weight gain, only one of the three rice cultures tested had adverse effects, causing depression, huddling, roughening of the coat and humped backs within 4–6 days at dietary levels of 25–50%. The 25% feed killed half of the mice within 3 weeks. The fungal mat of this isolate reduced weight gain and caused high mortality in mice given levels of 2·5% or more in the diet, and similar effects were produced with slightly higher concentrations of the other fungal mat tested. Some of the affected mice were jaundiced and all showed similar renal and hepatic lesions. Small green to yellow foci were visible in the liver and the predominant histopathological changes were bile-duct inflammation and disseminated hepatic-cell necrosis in the portal areas. In mice affected less acutely, the lesions were limited to hyperplasia and hypertrophy of the bile-duct epithelium, periductal oedema and fibrosis and mild inflammatory-cell infiltration. In the kidneys, mild tubular degeneration and necrosis were the usual reactions.

The liver lesions seen in this study were the same as those caused by *P. viridicatum*, which is known to be able to produce ochratoxin, but thin-layer chromatography failed to demonstrate OA in the *P. ochraceum* rice cultures and fungal mats, although this toxin was readily detected in similar cultures of *A. ochraceus*. Aflatoxin was also clearly absent, and citrinin, another metabolite of *P. viridicatum*, is primarily toxic to the kidney rather than the liver. The question of the metabolite responsible for the toxicity of *P. ochraceum* cultures is therefore still open, but it seems that, in this case at least, some compound other than the known ochratoxins is the culprit.

RECENT STUDIES ON LUNG PATHOLOGY IN SMOKERS

Lung cancer and chronic bronchitis are well-known hazards of cigarette smoking (*Cited in F.C.T.* 1970, **8,** 570). The pathogenesis of these lesions has been studied in some depth

in the past but attention to cytopathology has thrown fresh light on the pulmonary damage involved.

It is now realized that cigarette smoke has an adverse effect not only on the epithelial cells of the trachea and bronchi but also on other types of exposed cells, of which the pulmonary macrophage is one. Pratt *et al.* (*Lab. Invest.* 1971, **24,** 331) obtained human pulmonary macrophages by the new technique of endobronchial lavage and compared the morphology of such cells collected from smokers and non-smokers. The macrophages from non-smokers displayed large polymorphic nuclei, an extensive Golgi apparatus and various inclusions. The latter were round or oval, measured from 0·1 to 2·0 μm, were surrounded by a clearly defined membrane and exhibited acid-phosphatase activity. Such structures conform to the known character of lysosomal organelles in other species. A few of these lysosomal structures from non-smokers contained myelin-like figures and homogeneous lipid-like zones. In smokers, the inclusions were much more prominent, measuring up to 20 μm in diameter, and a higher proportion contained lipid-like zones. Moreover, dense angular and needle-like structures were observed only in the inclusions of smokers and it was thought that these possibly represented undigested smoke products.

Changes in pulmonary macrophages have also been described in dogs subjected to cigarette smoking (Frasca *et al. Expl mol. Path.* 1971, **15,** 108). The macrophages in tissue sections from the lungs of the smoking dogs were more numerous and more electron-dense than those present in untreated control dogs and possessed an irregular nucleus containing compacted chromatin. They may have been dislodged from the basement membrane, which was missing in some areas and thickened in others. The alveolar walls beneath the macrophages were considerably thickened and consisted mainly of fibroblasts and collagen. Collagenous thickening also occurred in the pleura. Small granulomas were present in the connective tissue of these thickened areas. The macrophages within these lesions contained crystalline structures, and ferritin-like particles were found in lysosomes and phagosomes. These findings tempt one to speculate on the possibility that the macrophages obtained by endobronchial lavage may have been derived from such microscopic granulomatous areas rather than from the ordinary pulmonary macrophages.

Fibrosis of the lung, chronic bronchitis and emphysema are known to occur in many heavy smokers, but it now appears that they may also be afflicted by another chronic pulmonary disease. Stoloff & Victor (*Archs envir. Hlth* 1972, **25,** 415) were struck by the high incidence of carcinoma of the lung in sufferers from bullous lung disease. They found that the risk of primary lung cancer was 61/1000 in men with bullous lung disease compared with 1·9/1000 in men without bullous disease. Bullous disease occurred only rarely in women and was much more common in men over 45 years old than in younger men and in non-white than in white men. Superimposed on these differences, however, was found a strong correlation between the disease and smoking. Stoloff & Victor (*loc. cit.*) found a bullous disease incidence of 2·5/1000 among 23,356 men over 24 years old (and only one case in 21,531 women) and in a sample covering 5% of the males studied, no case of the disease was found in a non-smoker. It was also found that the incidence rate was higher in men who were occupationally exposed to the inhalation of hazardous materials and particularly in those who recalled the occurrence of respiratory symptoms during such exposure.

Fortunately, despite the severity that chronic lung disease may attain in heavy cigarette smokers, the pathological process may be arrested and in some instances ameliorated in people who stop smoking. In a survey carried out in a telephone company among male employees aged between 40 and 59 years, Comstock *et al.* (*ibid* 1970, **21,** 50) found that

cigarette smokers had considerably more cough, phlegm and chronic wheeze and slightly more nasal catarrh and breathlessness on exertion than non-smokers. These respiratory symptoms were most common among heavy smokers and smokers of non-filter cigarettes. They were less marked among smokers of pipes and cigars than among cigarette smokers as a whole. The same employees were examined 6 years later, and men who had given up cigarette smoking between the two surveys showed considerable improvement in the degree of cough and phlegm. Furthermore, the decrease in forced expiratory volume (FEV) in this group between the two surveys was comparable to that in non-smoking controls and was consistent with the decline that occurs naturally between the age of 40 and 60 years. On the other hand, those who continued smoking showed a much more marked decrease in FEV and an exacerbation of the respiratory symptoms they exhibited 6 years earlier.

Smoking has been known to exacerbate chronic lung disease from other causes. In this context attention has been paid to its role in the pathogenesis of coal-workers' pneumoconiosis following reports that bituminous-coal workers who smoked cigarettes had more dyspnoea, persistent cough, ventilatory impairment and abnormal alveolar gas transfer than did non-smoking miners. However, these investigations were not very successful, at least partly because the radiological evidence many of the investigators employed for the studies was not found to be particularly helpful. Naeye *et al.* (*ibid* 1971, **22,** 190) approached the problem from a different angle. Using quantitative techniques on post-mortem material, they found that while in anthracite-coal miners there was no significant difference between smokers and non-smokers in the incidence and severity of emphysema or in the ratio of goblet cells to other lining cells in the peripheral airways, these criteria were consistently higher in bituminous-coal miners who smoked cigarettes than in those who did not. In neither the smoking nor the non-smoking groups was the collagen content of the coal-dust macule (fibrotic granuloma) increased. There is thus some concrete evidence that smoking adversely affects the pulmonary condition induced in coal-miners by the dust arising from cutting soft coal.

The weight of evidence implicating smoking as a factor in chronic lung disease has led some investigators to seek some means of protecting the lung mucosa from the irritant effect of cigarette smoke. A number of agents have been tried in the past, without much success, and a new compound has now been evaluated for this purpose by Jones *et al.* (*Br. med. J.* 1972, **2,** 142). Exposure of rats to 25 cigarettes a day for 24 days produced a basal cell and goblet-cell hyperplasia, which is accepted as an indication of the irritant effect of smoke on the respiratory epithelium. Addition of phenylmethyloxadiazole to the tobacco reduced these histological changes and in addition reduced the severity of the distress that otherwise was an immediate consequence of exposure.

The search for ways of reducing the irritancy of tobacco smoke has not been limited to means of modifying the smoke. A number of workers have looked into the possibility that the method of drying tobacco may have a considerable influence on the composition of the smoke it produces. Passey *et al.* (*ibid* 1971, **4,** 198) demonstrated that smoke from air-cured tobacco was much less liable to cause death to rats from respiratory damage than that from flue-cured tobacco. Flue-cured tobacco retains most of the natural sugars from the tobacco plant while the air-cured varieties lose most of this sugar by enzymatic degradation. The flue-cured tobacco also has a higher tar content than its air-cured counterpart and produces an acid smoke. Parallel studies on the two types of tobacco involving the painting of each type of tar on to the shaved backs of mice showed that, although possibly more irritant

to the skin, flue-cured tobacco produced fewer skin tumours than the air-cured form, which contained more "chemically recognized carcinogens".

The implication that the degree of harm caused by cigarette smokes depends on some constituent of the tobacco, possibly sugar, has been seriously questioned (*Lancet* 1973, **i,** 187) and it seems likely that differences in method of smoking, and particularly whether the smoke is inhaled (Stuttaford, *ibid* 1973, **i,** 680), are prime factors in the development or absence of lung damage.

There appears to be little evidence of any rapid progress towards the provision of a 'safe' cigarette. However, the search for substances that will reduce the irritant effect of smoke on the lung mucosa is a most appropriate line of research. Equally important is a greater understanding of the various ways in which cigarettes may habitually be smoked and the role played by these habits in determining the form or extent of any subsequent lung pathology.

Fd Cosmet. Toxicol. Vol. 11, pp. 909–932. Pergamon Press 1973. Printed in Great Britain

TOXICOLOGY: ABSTRACTS AND COMMENTS

EMULSIFIERS AND STABILIZERS

2577. Toxicology of xanthan gum

Woodard, G., Woodard, M. W., McNeely, W. H., Kovacs, P. & Cronin, M. T. I. (1973). Xanthan gum: Safety evaluation by two-year feeding studies in rats and dogs and a three-generation reproduction study in rats. *Toxic. appl. Pharmac.* **24,** 30.

Xanthan gum, a polysaccharide gum derived from *Xanthomonas campestris* and containing D-glucose, D-mannose and D-glucuronic acid as the predominant hexose units, is used in foods, cosmetics and pharmaceuticals as a thickening, suspending and stabilizing agent. Some of the studies that established the safety-in-use of gums of this type have now been published.

In a 2-yr study in rats, groups fed dietary levels providing daily doses of 0·25, 0·50 or 1·0 g/kg body weight showed no significant differences from controls in respect of survival, body-weight gain, haematology, blood-glucose levels and serum glutamic–pyruvic transaminase activity. Autopsy findings and histology were also comparable in the control and treated groups and there were no significant differences in tumour incidence. Soft stools occurred more frequently in the males given 0·5 or 1·0 g/kg/day than in the other groups but the differences were barely statistically significant. The feeding of xanthan gum in the diet to provide intakes of 0·25 or 0·50 g/kg/day by rats in a three-generation reproduction study was without effect on parental survival and reproductive performance or on litter size and condition, weights at birth and weaning, survival at weaning and gross autopsy observations. In each generation, the body weights of treated parents were slightly lower than those of the control parents.

In dogs, the gum was found to be similarly innocuous. Daily intakes of 0·25–1·0 g/kg fed for 107 wk had no significant effects on behaviour, body weights, haematology, blood biochemistry measurements, electrocardiograms, blood pressures, heart rates, neurological examinations, autopsy findings, organ weights or histopathology in either sex. The incidence of soft stools was more marked in the dogs than in the rats and was reflected in a dose-related increase in faecal weight and a compensatory increase in the specific gravity of the urine, together with an increased occurrence of urinary albumin in the dogs on the highest dose of the gum. The production of soft stools was attributed to the physico-chemical properties of xanthan gum.

PRESERVATIVES

2578. Aspects of nitrite toxicity

Sleight, S. D., Sinha, D. P. & Uzoukwu, M. (1972). Effect of sodium nitrite on reproductive performance of pregnant sows. *J. Am. vet. med. Ass.* **161,** 819.

Friedman, M. A., Greene, E. J. & Epstein, S. S. (1972). Rapid gastric absorption of sodium nitrite in mice. *J. pharm. Sci.* **61,** 1492.

Henderson, W. R. & Raskin, N. H. (1972). "Hot-dog" headache: Individual susceptibility to nitrite. *Lancet* **ii,** 1162.

Nitrite is known to produce toxic effects in animals by converting haemoglobin into methaemoglobin (*Cited in F.C.T.* 1968, **6,** 534), but there is conflicting evidence concerning its effect on reproductive capacity (*ibid* 1969, **7,** 386).

The first paper cited above describes experiments in which sodium nitrite was injected sc in single or multiple doses of 21–35 mg nitrite/kg into six sows in the first quarter of gestation and into nine others in the last third. Multiple (two or three) doses were given on consecutive days. Three sows died after doses of 30 mg/kg or more, their death being associated with severe convulsions. All the treated sows showed varying degrees of ataxia, weakness, dyspnoea and frequency of urination, but in only one did foetal death occur. The severity of toxic signs was paralleled by the proportion of haemoglobin converted to methaemoglobin in the sows, but little or no foetal haemoglobin was converted, suggesting that the nitrite did not cross the porcine placental barrier to any appreciable extent. Data obtained during hysterotomy of sows given sc or iv doses of nitrite confirmed this protective function of the placenta but, as was to be expected, the highest maternal methaemoglobin concentrations caused a marked decrease in the oxygen saturation of the foetal blood. The authors conclude that acute nitrite toxicosis is unlikely to be a factor in swine abortion, since a dose that does not prove lethal to the sows is unlikely to affect the piglets.

The second paper reports that 85% of an oral dose of sodium nitrite disappeared from the mouse stomach within 10 min. After 30 min, 95% of the dose had disappeared. This rapid loss was not inhibited by ligature of the gastro-duodenal junction. *In vitro* incubation of nitrite solutions in isolated stomachs showed losses of 35% in 10 min and 63% within 30 min, although no absorption was possible. However, the rate of disappearance under these conditions was much slower than that *in vivo* and it was demonstrated that 40% of the gastric nitrite had been converted to nitrate. It is evident from these findings that direct absorption of nitrite from the stomach contents into the blood is the major factor in its removal from the alimentary tract in the mouse. Similarly rapid absorption was also indicated in earlier studies in several other species and may be a limiting factor in the suggested biosynthesis of nitrosamines in the gut.

Unusual human susceptibility to nitrite is the subject of the third paper cited above. This describes the case of a man who found that he developed headaches shortly after consuming frankfurters. When he was subjected to a series of tests to determine whether the nitrite in frankfurters was responsible for this reaction, unidentified drinks containing 10 mg or less of nitrite provoked a headache on eight out of 13 occasions. He also developed a headache after taking 100 mg tyramine hydrochloride, but not after drinking sodium bicarbonate placebos. The results suggest the possibility that nitrite and tyramine may affect a common humoral mediator or locus of action. Many foods produce adverse reactions in a few unfortunate individuals and a variety of constituents are undoubtedly involved. Tyramine, histamine and other amines have been held responsible for some other postprandial headaches (*Cited in F.C.T.* 1973,**11,** 343). Fortunately the particular reaction now reported seems, like most of the others, to be a rare phenomenon.

2579. Sulphite and the stomach

Feron, V. J. & Wensvoort, P. (1972). Gastric lesions in rats after the feeding of sulphite. *Pathologia Eur.* **7,** 103.

There are lingering doubts over the possible toxicity of sulphites in food. Added freshly to diets, sulphite has proved relatively harmless to animals, apart from its known inactivation of dietary thiamine, but interference with nucleic acid synthesis *in vitro* has suggested that there may be more in the picture than appears at first glance (*Cited in F.C.T.* 1973, **11,** 504). The effects on rats of feeding thiamine-enriched diets containing high levels (0·5–8%) of sodium metabisulphite (I) for 10–56 days, and of feeding 0·125–2% I for up to 2 yr have now been reported.

At dietary levels of 1% or more, I produced a thickening of the limiting ridge of the forestomach, while at levels of 6–8% an irregularly shaped thickened ridge overhanging the glandular part of the stomach was formed. Microscopically, the thickening was seen to be due mainly to severe local hyperkeratosis and acanthosis. A few animals showed papillomatous growths. The long-term trial produced, in general, no more pronounced lesions than appeared in the short-term trial with corresponding dietary levels of I.

In most of the rats fed 4–8% I in the shorter test, the fundus of the glandular stomach developed haemorrhagic erosion, epithelial necrosis, eosinophilic infiltration and some glandular hyperplasia. About 30% of rats fed 2% I for 2 yr developed a mild atrophic gastritis. There was no evidence in either trial of any damage in the pyloric region of the stomach.

AGRICULTURAL CHEMICALS

2580. Long-term feeding study on DDT

Tomatis, L., Turusov, V., Day, N. & Charles, R. T. (1972). The effect of long-term exposure to DDT on CF-1 mice. *Int. J. Cancer* **10,** 489.

There is much inconclusive evidence relating to the alleged carcinogenicity of DDT in animals. In an attempt to clarify the situation and in response to a recommendation expressed in 1967 by the FAO/WHO group concerned with pesticide residues, a large-scale investigation of the potential carcinogenicity of DDT in rodents was initiated by the International Agency for Research on Cancer in collaboration with several national institutes. The experiments in mice have been planned to continue for seven consecutive generations and an interim report has now appeared reporting the results for the parent and first-generation offspring.

In the part of the experiment so far completed, technical DDT was fed to mice of both sexes at levels of 2, 10, 50 and 250 ppm in the diet throughout the lifespan of two generations. One-third of the 60 females in each group were mated at 9–10 wk to produce the F_1 generation. At levels below 250 ppm, DDT had no significant effect on pre-weaning mortality, which was, however, greatly increased in the group fed 250 ppm. Adult mortality was also higher in this group than in the others, the difference becoming apparent from about wk 60. Signs of DDT intoxication (restlessness, tremors and convulsions) occurred in one F_1 female

given 50 ppm DDT and in 16 females and six males of the parent generation and three females and one male of the F_1 generation fed 250 ppm DDT. These responses were generally apparent only in mice over 70 wk old.

Liver tumours were more numerous in the males of all the four groups treated with DDT than in those of the control group. The increase was most marked in the 250 ppm group, in which the incidence was also significantly increased in females. Only a slight increase in liver tumours was seen in females fed 10 to 50 ppm DDT. The percentages of tumour-bearing animals (based in each group on the number surviving when the first tumour was observed at any site) were, in males and females respectively, 84 and 80% in the P generation and 62 and 85% in the F_1 generation of the untreated controls, 88 and 90% (P generation) and 92 and 95% (F_1 generation) in the group given 250 ppm DDT and 96 and 93% (P generation) and 97 and 97% (F_1 generation) in a urethane-treated positive-control group. In the latter group, the increase in tumour incidence was associated only with lung tumours. The incidence of tumours at sites other than the liver did not appear to be affected by DDT treatment.

Liver tumours generally appeared at an earlier age in the DDT-treated animals than in untreated controls and also appeared earlier in the F_1 than in the parent generations of the DDT groups. The age at which the mice with liver tumours died, as well as the incidence of such tumours, appeared to be related to the dose of DDT. Only four of the liver tumours metastasized, and although they all occurred in DDT-treated animals the small number involved prevented any conclusion being drawn from this. Histologically the liver tumours took the form of well-differentiated nodular growths, either pressing on the surrounding liver parenchyma without infiltration or obliterating the liver structures and forming glandular or trabecular patterns.

2581. Oestrogenic effects of DDT

Clement, J. G. & Okey, A. B. (1972). Estrogenic and anti-estrogenic effects of DDT administered in the diet to immature female rats. *Can. J. Physiol. Pharmac.* **50,** 971.

We referred recently to the oestrogenic and anti-oestrogenic properties of DDT in connexion with a demonstration that this insecticide can prolong the reproductive life of ageing female rats (*Cited in F.C.T.* 1973, **11,** 690). The paper cited above is concerned with its effect on female rats at the other end of their life cycle.

Immature female Wistar rats were fed diets containing 500–3000 ppm *o,p'*-DDT from days 23–30 after birth and were then killed for determination of uterine wet and dry weights and protein and glycogen levels. Diethylstilboestrol (DES) at a level of 100 ppb ($b = 10^9$) was given to a positive control group, while other rats received the basic diet alone. The administered DDT had a clearly oestrogenic effect, producing increases in uterine weights, protein and glycogen. The dose response was linear over the range tested. The increases were statistically significant, however, only at dietary levels of 1000 ppm or more. All the DDT levels caused some premature vaginal opening, a sensitive indicator of oestrogenic activity but again the effect was only statistically significant at 1000 ppm and above. All the open vaginas contained the cornified epithelial cells characteristic of mature females during the onset of oestrus. A lower dietary level of *o,p'*-DDT (100 ppm) given over the same period significantly reduced the oestrogenic effect on uterine glycogen exerted by oestradiol injected sc from day 23 in a dose of 0·5 μg/day, probably by stimulating hepatic

degradation of the steroid. The intrinsic oestrogenic activity of DDT at levels of 500–1500 ppm, however, brought the glycogen response in the oestradiol-treated rats much nearer to that produced by oestradiol alone or by oestradiol and 5–50 ppm DDT.

In a similar series of experiments, *p,p'*-DDT was shown to lack significant oestrogenic activity, but at 100 ppm in the diet it counteracted oestradiol-stimulated advancement of vaginal opening.

The authors consider that, in view of the high levels of DDT intake associated with oestrogenic activity, it is unlikely that mammals or birds could ingest oestrogenic levels even in grossly contaminated areas. They suggest that if mammalian reproduction were compromised by DDT intake, it would be more likely to be due to an acceleration of steroid degradation.

2582. DDT and vitamin A in man

Keil, J. E., Sandifer, S. H., Finklea, J. H. & Priester, L. E. (1972). Serum vitamin A elevation in DDT exposed volunteers. *Bull. env. contam. & Toxicol.* (*U.S.*) **8,** 317.

It has previously been reported that DDT ingestion can lead to reduced liver storage of vitamin A in rats, though the mechanism involved was in some doubt (*Cited in F.C.T.* 1964, **2,** 257). High DDT intakes (dietary levels of 40–60 ppm) by yearling beef steers caused some depletion in liver stores of vitamin A and increased the serum levels of the vitamin, but the changes were relatively small (Phillips & Hidiroglou, *J. agric. Fd Chem.* 1965, **13,** 254). The present paper reports the results of another study in which the findings in 21 volunteers who had experienced occupationally a considerable degree of exposure to pesticides were compared with those in a control group of another 21 volunteers matched by age, race and sex but with no known pesticide exposure.

Fasting blood samples were taken for DDT and vitamin A analyses. Blood for vitamin A determinations was wrapped in aluminium foil to prevent photodegradation. The pesticide-exposed group had significantly higher serum levels of vitamin A, DDT, DDD and DDE than their matched controls, although the vitamin A levels of both groups were within the normal range. There was a significant positive linear correlation between levels of vitamin A and of DDT and its principal metabolites, but this could have been a natural consequence of the lipid solubility of these compounds.

The raising of serum levels in association with a decrease in liver stores of vitamin A may be inferred from this as from previous studies, but this could be demonstrated conclusively only by serial sampling of sera in conjunction with liver biopsy. It is not certain whether the additional vitamin A in the serum is mobilized from reserves in the liver or whether its absorption from the blood is restricted.

2583. Organochlorine residues in human blood

Mick, D. L., Long, K. R. & Bonderman, D. P. (1972). Aldrin and dieldrin in the blood of pesticide formulators. *Am. ind. Hyg. Ass. J.* **33,** 94.

Siyali, D. S. (1972). Hexachlorobenzene and other organochloride pesticides in human blood. *Med. J. Aust.* **2,** 1063.

A review of studies on organochlorine residues in the environment and of methods of assessing occupational exposure to such compounds suggested some years ago that although pesticide residues did not appear to constitute a threat to public health, vigilance would

continue to be necessary to detect any indication to the contrary (*Cited in F.C.T.* 1971, **9,** 425).

The first paper cited above compares the aldrin and dieldrin concentrations in the blood of 21 employees who had formulated some 2000 lb of technical aldrin with the levels in the blood of other workers who had been involved in the formulation of chlorophenoxyacetic acid herbicides (2,4-D and 2,4,5-T). Mean plasma levels of aldrin ranged from 0·8 ppb ($b = 10^9$) in the herbicide handlers to 29·5 ppb in the pesticide formulators, and mean dieldrin levels (derived from aldrin by epoxidation) were 11 and 182·5 ppb, respectively, in these two groups. Erythrocyte concentrations of aldrin varied from zero in the herbicide handlers to 3·7 ppb in the pesticide formulators. As exposure to aldrin increased, residues of aldrin and dieldrin in the plasma rose more rapidly than those in the erythrocytes. Thus the main indicators of aldrin exposure appear to be increases in plasma dieldrin and erythrocyte aldrin.

The second paper cited considers the organochlorine residues detected in the whole blood of 237 residents of New South Wales, 185 of whom had a history of occupational exposure to organochlorine pesticides. More than 95% of the whole group were found to have detectable levels of hexachlorobenzene (HCB) in their blood. This fungicide has not been widely reported in human tissues, although its misuse has led to some outbreaks of poisoning. Other residues detected in the blood samples in the present study included aldrin, dieldrin, lindane, other benzene hexachloride isomers, DDT and heptachlor epoxide, in concentrations similar to those reported elsewhere in the world. These compounds occurred in about the same proportion of cases in the exposed and non-exposed groups, but the actual levels were expectedly somewhat higher in the former group. Analysis of various foods, notably animal fats and eggs, indicated foods as a source of some of the pesticides, particularly HCB, DDT and dieldrin. Neither of the study groups showed any sign of intoxication, and the levels involved have no known significance. It is noted, however, that blood levels of 100 ppb DDT, 50–100 ppb HCB, 20 ppb BHC, heptachlor epoxide and lindane or 15 ppb dieldrin possibly indicate either recent exposure in excess of normal environmental quantities or the mobilization of pesticides from rat depots as a result of a general loss in body weight.

PROCESSING AND PACKAGING CONTAMINANTS

2584. Transfused phthalate from PVC blood bags

Jaeger, R. J. & Rubin, R. J. (1972). Migration of a phthalate ester plasticizer from polyvinyl chloride blood bags into stored human blood and its localization in human tissues. *New Engl. J. Med.* **287,** 1114.

Brief mention of some of the work of these authors was made in September 1972 at Pinehurst, N.C., at a meeting on phthalate esters. This paper is concerned specifically with diethylhexyl phthalate (DEHP) and its passage from blood-storage bags made of plasticized polyvinyl chloride (PVC) to the tissues of patients given transfusions of the stored blood.

Studies of the migration of DEHP into human and dog blood stored for up to 21 days at 4°C in blood bags made from PVC containing, on average, 37% DEHP demonstrated

in both types of blood a linear accumulation of the plasticizer with increasing time of storage. The rate of accumulation was about 0·25 mg/100 ml/day, so that by day 21 DEHP levels in human blood had reached 5–7 mg/100 ml. The method of analysis used detected no DEHP in the human blood before storage. Fractionation of human blood stored for 14 days showed that the bulk of the DEHP was present in the lipoprotein fraction of the plasma (density $< 1{\cdot}21$), but appreciable quantities were found also in the more dense plasma fraction and in the washed red cells.

The fate of the plasticizer in patients given blood stored in PVC bags was studied by analysing tissue samples (lung, liver, spleen and abdominal fat) obtained at autopsy from patients who died after receiving blood transfusions. In some cases, their blood had also been in contact with PVC tubing in a cardiopulmonary-bypass unit. The results were very variable, DEHP being found in tissues in some patients but not in others, but lung tissue from seven out of twelve transfused patients contained detectable amounts (13–91 μg/g dry weight of tissue). A very high level (270 μg/g) of DEHP in the abdominal fat of one obese patient with pancreatitis remained unexplained. No detectable levels were found in this fat from the rest of the transfused patients or from others who died without receiving transfusions.

The demonstrated variation in tissue retention prompted a study of the possible excretion of phthalic acid by a patient given open-heart surgery. Urine was collected for 2 days before surgery, on the day of operation and for the following 5 days. It was calculated that during surgery he received 21 mg DEHP in transfused blood, and his blood was also exposed to PVC tubing for 1·5 hr during cardiac bypass. Assay of the urine samples showed a 24-hr total of some 8 mg phthalic acid (free and as glucuronides, sulphates and monoesters) on both of the days before surgery. This could not be explained, but during the 24 hr in which transfusions were given, the increase in urinary phthalic acid excretion represented 43·3 mg DEHP, thus accounting for the plasticizer administered by transfusion and suggesting a further uptake of 22 mg from the bypass tubing. On the following 5 days, the urinary output of DEHP was no greater than on the days before transfusion. It seemed, therefore, that this patient was capable of metabolizing and rapidly excreting the phthalate administered.

The authors of this report calculate that in a 70-kg man a whole-body exchange transfusion with 21-day-old blood could result in the iv administration of approximately 300 mg DEHP, or a dose of 4–5 mg/kg body weight.

2585. The fate of diethylhexyl phthalate in the rat

Albro, P. W., Thomas, R. & Fishbein, L. (1973). Metabolism of diethylhexyl phthalate by rats. Isolation and characterization of the urinary metabolites. *J. Chromat.* **76,** 321.

In spite of the widespread use of phthalate esters and current concern about certain aspects of their presence as environmental contaminants, relatively little is known about their metabolism in man and other species. There is some evidence for the partial hydrolysis of orally administered diethyl and dibutyl phthalates in the rat, resulting in excretion of the monoester and free acid in the urine (*Cited in F.C.T.* 1972, **10,** 724), but in the dog, oral doses of butylbenzyl phthalate are largely excreted in the faeces and both dog and man excrete little urinary phthalic acid after an oral dose of di-2-ethylhexyl phthalate (DEHP). More recent work in rats, using DEHP labelled with ^{14}C in the carbonyl groups, indicated that the radioactivity was rapidly eliminated in the urine and faeces. Following oral administration, the distribution of ^{14}C excretion was about 55% in the urine and 35% in the

faeces, while after iv injection the proportion appearing in the urine was greater (Schulz & Rubin, Phthalic Acid Esters Conference, NIEHS, Pinehurst, N.C., 6–7 September 1972).

A study is now reported in which rats were given two intubated doses of 0·2 ml [^{14}C]-DEHP 24 hr apart. Urine was collected for 48 hr after the first dose and was subjected to thin-layer chromatography and, after treatment with diazomethane, to thin-layer and gas–liquid chromatography. The metabolites isolated in this way were characterized by infra-red and nuclear magnetic resonance spectroscopy and mass spectrometry. It appeared that the DEHP metabolites were not conjugated prior to excretion. Free phthalic acid accounted for less than 3% of the urinary metabolites, but was the only detectable ^{14}C-labelled product after alkaline hydrolysis of either the total ether extract of the urine or the individual metabolites. No mono-2-ethylhexyl phthalate (MEHP) was present in the urine, but the five metabolites identified suggested that hydrolysis of the administered DEHP to MEHP was followed by ω-oxidation and (ω — 1)-oxidation, probably in the liver, with the alcohol intermediates being further oxidized to the level of ketone after (ω — 1)-oxidation or acid after ω - oxidation and the resulting acid metabolite then undergoing β-oxidation. It thus appears that, in the rat, DEHP is handled like a fatty acid, ω-oxidation being the main metabolic pathway since α- and β-oxidation are initially impossible.

2586. Another check on ethylene oxide sterilization

Lawrence, W. H., Dillingham, E. O., Turner, J. E. & Autian, J. (1972). Toxicity profile of chloroacetaldehyde. *J. pharm. Sci.* **61,** 19.

Mantz, J. M., Tempe, J. D., Jaeger, A. et Vidal, S. (1972). Sténoses trachéales et stérilisation des canules de trachéotomie par l'oxyde d'éthylène. *Sem. Hôp. Paris* **48,** 3367.

Ethylene chlorohydrin (EC) is a reaction product occurring in plastics, spices and foods containing chloride and sterilized by exposure to ethylene oxide (EO) (*Cited in F.C.T.* 1972, **10,** 592). One metabolite of EC in rats has been found to be chloroacetaldehyde (CA), so that a clear picture of the potential hazard of CA should be of assistance in any assessment of the possible results of inadequate removal of EO from sterilized products.

The LD_{50} of CA by ip injection was found to be 6 μl/kg in mice, 6–8 μl/kg in rats, 2 μl/kg in guinea-pigs and 5 μl/kg in rabbits. When administered orally by intubation, its LD_{50}s in mice and rats were 69 and 75–86 μl/kg, respectively, while its dermal LD_{50} in rabbits was 224 μl/kg. When mice were exposed to a CA–air mixture in an inhalation chamber, the time required to kill 50% of the mice (LT_{50}) was 2·57 min, at which time the chamber atmosphere had reached approximately 45% of equilibrium with the incoming mixture, produced by bubbling air through 30% aqueous CA. CA was extremely toxic to mouse fibroblast cultures, inducing 50% inhibition of protein synthesis at $5{\cdot}62 \times 10^{-5}$ M concentration. It also proved very irritant to the skin and eyes of rabbits. Plastics implants previously treated with CA produced muscle necrosis in rabbits. Pretreatment of mice with CA, either by inhalation or ip injection, resulted in a dose-related prolongation of pentobarbitone sleeping time, an effect which had no apparent association with hepatic necrosis. When injected iv, CA reduced the blood pressure of anaesthetized rabbits, had a variable effect on their respiration and, in large doses, inhibited neuromuscular transmission. Repeated ip injection of 1·6 or 3·2 μl/kg thrice weekly for 12 wk induced chronic focal bronchopneumonia and associated morphological alterations of the respiratory epithelium suggestive of a premalignant condition.

Comparison of these results with others already obtained for EC (*ibid* 1972, **10,** 589) indicates that while the two compounds are similar in their acute oral toxicity in rats and mice, CA is some 10–30 times more toxic than EC when injected ip. Conversely EC is more toxic than CA following application to the skin. CA is by far the more irritant of the two compounds, however.

The second paper cited above reports an abrupt increase during 1969 in the incidence of tracheal stenosis in patients subjected to tracheotomy and prolonged artificial respiration, and attributes this to the inefficient removal of residues from cannulae sterilized with EO 48 hr before use. When the storage period between EO sterilization and use was increased to 15 days, the incidence of tracheal stenosis fell again to the levels recorded before EO sterilization was instituted as a routine measure. Storage of sterilized equipment of this kind for at least 8 days after exposure to EO is recommended as a safety precaution.

2587. Testing of plastics for pharmaceutical use

Lefaux, R. (1972). Valeur et limites d'un contrôle analytique des matières plastiques à usage pharmaceutique et médical. *Annls pharm. fr.* **30,** 673.

Pentelow, J. E. (1973). Practical problems in the examination and control of plastics for pharmaceutical use. *Proc. Soc. analyt. Chem.* **10,** 41.

The increasing use of plastics containers for pharmaceutical products raises the problem of the analytical testing of these materials. Two authors, one French and one English, have recently presented reviews on this subject and show some degree of accord in their general approach.

The test methods for the assessment of plastics reviewed by Lefaux (first paper cited above) fall into three general categories covering tests on the plastics material itself, tests on extracts prepared by autoclaving the plastics, usually in contact with distilled water, and biological testing. The first category covers the physico-chemical properties of the plastics material (permeability to light, ultraviolet rays, air, water vapour, bacteria and viruses and absorption properties), and when these have been checked the polymer itself can be examined by a variety of methods involving extraction by various solvents, chromatographic separation and analysis by ultraviolet, infra-red, mass spectrometric and nuclear magnetic resonance methods. The polymer should be subject to routine analytical control on the basis of its infra-red spectrum and the extraction of additives.

The autoclaved extract is tested for organic materials, heavy metals, tin, zinc and chromium, and can be subjected to spectrophotometric analysis. These tests may give an adequate analysis of the polymer itself, but biological testing is still very important and the author points out that there is room for improvement in current methods. He stresses the potential value of adding more sensitive procedures, such as cytotoxicity and haemolysis tests, to the acute toxicity tests in mice. The risk of accumulation of substances that are not metabolized is probably a more frequent problem than that of acute toxicity.

The second paper cited above again reviews existing test procedures, laying particular stress on the testing of the final container. The testing of the physico-chemical properties of the plastics and the monitoring of extracts are described, with reference to the methods approved by those authorities who have produced monographs or guidelines on this subject. On the same basis, the author restricts his attention to the biological tests already recommended, without speculating on possible advantageous additions to the list. Consistent quality control of the plastics is again advocated.

[Both authors outline a general principle for plastics control consisting of careful assessment of the polymeric material and its additives and of extracts from the formed polymer, and both stress the importance of biological testing, although differing types of test are proposed within this framework. However, it is interesting to note that, as the author of the second paper comments, the overall picture in this field has not changed appreciably since the mid-1960s (*Cited in F.C.T.* 1965, **3,** 531).]

THE CHEMICAL ENVIRONMENT

2588. Mechanism of carbon disulphide neurotoxicity

Tarkowski, S. & Cremer, Jill E. (1972). Metabolism of glucose and free amino acids in brain, studied with ^{14}C-labelled glucose and butyrate in rats intoxicated with carbon disulphide. *J. Neurochem.* **19,** 2631.

Over-exposure to carbon disulphide (CS_2) can produce a long list of adverse effects, many of which are associated with the central or peripheral nervous system. In the past, some authors have suggested that this neurotoxicity was due to the interference of CS_2 with oxidative processes, such as mitochondrial oxidative phosphorylation (*Cited in F.C.T.* 1973, **11,** 151), and the present study was aimed at a further investigation of processes associated with energy metabolism in the brains of rats poisoned with this compound.

Rats exposed to an atmosphere of CS_2 (2·5 mg/litre) for 15 hr developed a significant hypoglycaemia, together with changes in the amino acid level of the brain. Notably, glutamine levels increased at the expense of glutamate, and γ-aminobutyrate (GABA) levels were markedly reduced. CS_2 did not, however, affect glycolysis or incorporation of ^{14}C from glucose into amino acids, as measured by iv injection of [2-^{14}C]glucose. When [^{14}C]-butyrate was the precursor, however, a different pattern of amino acid labelling was observed, exemplified by an enhanced ^{14}C incorporation into glutamine.

The authors conclude that their findings support the postulated two-compartment model according to which some of the brain glutamine is the precursor of the so-called 'large' glutamate pool while the rest gives rise to a 'small' glutamate pool. According to such a model, CS_2 merely affects metabolism associated with the 'small' pool, which includes the tricarboxylic acid cycle but excludes carbohydrate metabolism to pyruvate. It is proposed that the observed CS_2-induced changes may involve an increase in the rate of ammonia removal. The low incorporation of ^{14}C from either precursor used into GABA is compatible with previous findings that CS_2 interferes with the metabolism of vitamin B_6 through an effect on certain pyridoxal phosphate-dependent enzymes.

2589. Lead levels in blood, bone and water

Crawford, M. D. & Clayton, D. G. (1973). Lead in bones and drinking water in towns with hard and soft water. *Br. med. J.* **2,** 21.

Lead (Pb) values considerably above conventional safety limits (*Cited in F.C.T.* 1972, **10,** 228; *ibid* 1973, **11,** 498) have been reported in 'overnight' water from some consumers' taps, particularly in some soft-water towns (*ibid* 1969, **7,** 255; Beattie *et al. Br. med. J.* 1972, **2,** 491) and rural areas (Beattie *et al. ibid* 1972, **2,** 488), underlining the fact that this

toxic metal is leached from Pb plumbing systems more readily by soft than by hard water. The latter paper and a brief report by Williams (*ibid* 1972, **3,** 174) record Pb levels in the blood up to about 150 μg/100 ml among several rural families using Pb-piped water; clinical symptoms consistent with Pb poisoning were found in a number of the people involved. These studies suggested that domestic Pb pipes might constitute a more serious threat to health than has hitherto been recognized.

Taking advantage of the fact that Pb accumulates in the skeleton, efforts were made to determine the true extent of Pb exposure by studying the Pb content of the bones of people living in different areas. Preliminary studies had revealed that figures for the Pb content of rib bones from inhabitants of Glasgow, one of the major urban soft-water areas, were higher than those from people in London, which has hard water (Crawford & Crawford, *Lancet* 1969, **ii,** 699). In a subsequent investigation (Crawford & Clayton, cited above), Pb levels were determined in 39 'overnight' samples of tap-water from five hard-water towns, in 44 samples from five soft-water towns and in 13 samples from Glasgow. In addition, portions of ribs from victims of sudden death aged 30–60 yr were obtained from three towns supplied with hard water (27 rib samples) and three supplied with soft water (28 rib samples), and their Pb content was determined.

The mean Pb content of bones from all the soft-water towns was found to be 33·5 ppm dried ash, while that from the hard-water towns was 23·6 ppm. There was no overlap in the individual means for the two groups of towns. Pb levels in the drinking-water from the five hard- and five soft-water towns were, however, comparable, the mean for each type of area being 0·03 ppm, with only a very small proportion of samples in both groups exceeding 0·1 ppm. In Glasgow, where the water is particularly soft (with an average hardness of only 10 ppm $CaCO_3$ compared with 37 ppm in the other soft-water areas studied and 291 ppm in the hard-water towns), the Pb in nine of the 13 samples exceeded 0·1 ppm.

The higher Pb content found in the samples of bone from soft-water towns despite comparable Pb levels in the drinking-water was thought to be due to the fact that Pb ingested in hard water was only poorly absorbed because of the presence of large quantities of calcium. No further evidence was obtained in support of previous suggestions that absorption of Pb from drinking-water might be a factor in the reported differences between hard- and soft-water areas in mortality from cardiovascular disease.

[Despite the authors' attempt to explain the higher Pb content in bone from soft-water areas in terms of the more ready absorption of the Pb in soft water, it is by no means certain that other factors may not play a bigger role. The presence of atmospheric pollution from factories, for example, could perhaps account for this difference. Unfortunately the authors failed to look further afield than the drinking-water!]

2590. Intranuclear inclusions in lead poisoning

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

Chronic lead intoxication in man and animals is characterized by the presence of intranuclear inclusions in the proximal tubular epithelial cells of the kidney (*Cited in F.C.T.* 1973, **11,** 348; Goyer *et al. Archs envir. Hlth* 1970, **20,** 705). These inclusions do not appear to contain DNA or RNA but they contain protein, which is presumed to be of a non-histone type, and lead is also present. It has been thought that the development of these inclusions requires exposure to lead for 4–8 wk, but examination of the kidneys of rats injected ip

with an aqueous solution of lead acetate in a single dose of 50–200 mg/kg body weight has now shown that the inclusions appear as early as 24 hr after even the smallest of these doses. The inclusions, which were restricted to the proximal tubules, were detected in 10–25% of the epithelial cells 1 day after the injection, and the percentage of cells affected did not increase significantly with an increase in the dose of lead over a period of 6 days following treatment.

Inclusions were observed not only in the nucleus but also in the cytoplasm and presented a characteristic appearance under the electron microscope. The intranuclear inclusions consisted of a characteristic fibrillar mesh and dense amorphous material, the latter usually located in the interior of the inclusions. The fibrils varied in length (50–250 nm) and were about 12 nm thick. The cytoplasmic structures were similarly composed of fibrillar and amorphous material. In view of the rapid appearance of these intranuclear inclusions, the examination of renal biopsy specimens by electron microscopy may afford a means of detecting acute lead poisoning in man.

2591. Cerebellar target for lead in young rats

Thomas, J. A., Dallenbach, F. D. & Thomas, Manorama (1973). The distribution of radioactive lead ^{210}Pb) in the cerebellum of developing rats. *J. Path.* **109,** 45.

The brain is frequently involved in lead poisoning, especially in young children (*Cited in F.C.T.* 1964, **2,** 117), but although lead encephalopathy has been recognized for many years, the pathogenesis of the lesion is still obscure. An experimental model of lead encephalopathy has been developed (*ibid* 1969, **7,** 260) using newborn rats poisoned with lead that was fed to the mothers and transmitted to the young via the milk. The brains of newborn rats poisoned in this way showed capillary damage, severe oedema and haemorrhage, mainly in the cerebellum. This was confirmed by a later study (Thomas *et al. Virchows Arch. Abt. A path. Anat.* 1971, **352,** 61).

The fate of lead in the cerebellar tissue of newborn rats has now been investigated by means of light- and electron-microscope autoradiography, following the ip injection of 5 or 10 μCi radioactive lead (^{210}Pb) into day-old litters. Individual rats from each litter and from litters used as controls were killed at intervals between 1 and 168 hr after injection. Study of the light-microscope autoradiographs showed that up to 6 hr after injection radioactivity was confined almost entirely to the blood-vessel walls, but from 24 hr onwards there was some involvement of the neural parenchyma. By 168 hr, additional areas of patchy haemorrhage were apparent, with marked labelling of the extruded erythrocytes. Granules indicating the site of lead deposition were observed by electron microscopy in the endothelial cells of the capillaries of the cerebellum as early as 1 hr after the injection and were found in the same area in the 168-hr sections. Similar granules were noted at 72 and 168 hr in the astrocytes but their density was much lower than that in the endothelial capillaries. A few granules were also found over the oedematous spaces that were visible from 72 hr onwards.

These findings indicate that the primary target for lead deposition in the brain is the endothelial cell. The lead appears to pass through the intact endothelium to the brain cells.

2592. Targets for methylmercury

Herman, S. P., Klein, R., Talley, F. A. & Krigman, M. R. (1973). An ultrastructural study of methylmercury-induced primary sensory neuropathy in the rat. *Lab. Invest.* **28,** 104.

A peripheral sensory neuropathy was described in victims of the methylmercury (MeHg) poisoning incident at Minamata (Eyl, *Clin. Toxicol.* **4,** 291) and sensory nerve involvement has also been found in several species of animal treated experimentally with MeHg. These experimental effects were associated with histological evidence of sensory nerve degeneration and changes in the dorsal root ganglia and spinal cord.

The pathological changes have now been investigated further in a study in which adult rats were injected sc with MeHg hydroxide in daily doses equivalent to 2 mg Hg/kg on 5 days/wk for 1–3 wk. The animals were observed for up to 25 days after their last dose. During the first 13 days of treatment the animals gained little or no weight but showed no signs of neurological disturbance (stage I). The next stage (stage II; days 14–19) was characterized by a loss of weight amounting to as much as 20% of the initial body weight, but again no neurological effects were apparent. Continuing loss of weight and neurological symptoms became increasingly severe during stage III (days 20–24) and stage IV (day 25 to sacrifice). The symptoms consisted of disturbances of gait (ataxia) and marked crossing of the limbs when the animals were suspended.

Morphologically, changes were present in both the central nervous system and the peripheral nerves, notably the sural and sciatic nerves and the dorsal roots. The nerve changes during stage I consisted of slight enlargement of the Schwann cells and focal swelling of the myelin sheaths. During stage II, the nerve axons showed irregular varicosities, which progressed to the breakdown of myelin during stages III and IV. Changes were seen in the nerve cells of the dorsal root ganglia during stage II. These affected mainly the structures surrounding the nucleus and consisted of a loss of Nissl granules and vacuolation of the cytoplasm. Later on, particularly in stages III and IV, neuronal necrosis was observed. This occurred also in the brain (cerebellum, visual cortex and basal ganglia) in these terminal stages and was accompanied by hyperplasia of the astrocytes. In stages I and II, astrocytic hyperplasia was the only change observed.

These findings thus confirm the earlier reports and indicate that the primary targets of MeHg hydroxide in the peripheral nervous system are the sensory fibres and in particular the neurones of the spinal ganglia.

2593. Sensitivity to nickel in stainless steel

Barranco, V. P. & Solomon, H. (1973). Reactions to nickel. *J. Am. med. Ass.* **223,** 326.

A further round in the argument about the possibility of sensitization to nickel in stainless steel (*Cited in F.C.T.* 1973, **11,** 699) features a letter from the authors of the original article (Barranco & Solomon, *J. Am. med. Ass.* 1972, **220,** 1244).

They refute the suggestion that their patient's condition could have been due to primary irritation, on the grounds that he reacted positively to a patch test of chemical nickel in petrolatum and his dermatitis cleared promptly and completely only when the stainless steel screw was removed. They claim that there have been other reports of widespread eczematous dermatitis due to internal exposure to nickel in stainless-steel plates and suggest that while nickel is not detectable in the steel by the dimethylglyoxime spot test, some may well have been extracted by the body fluids in which the screw was steeped for 16 months. It is also suggested that while the minimum reaction time for cases of 'external' allergic contact dermatitis is generally accepted to be 12 hr, the reaction time may be much shorter in patients sensitized to a material in 'internal' contact.

[Having pointed out the arguments on both sides, we await further developments.]

2594. Placental transport of tellurium

Agnew, W. F. (1972). Transplacental uptake of ^{127m}tellurium studied by whole-body autoradiography. *Teratology* **6,** 331.

Tellurium (Te) given to rats at a dietary level of 500 ppm on days 10–15 of gestation has been shown to cross the placental barrier and cause hydrocephalus in the offspring (*Cited in F.C.T.* 1973, **11,** 337). Direct tissue counts and whole-body autoradiography have now been used to determine the specific localization of radioactive Te in rat foetuses at various stages of gestation following ip injection of the dams with a single dose of ^{127m}Te (0·25–0·35 mg/kg; 600 μCi) on day 7, 8, 9, 10, 11, 14, 15, 17 or 20. Each rat was anaesthetized 24 hr after the injection, maternal blood was sampled by cardiac puncture and the uterus was removed for study of the uterine horns, foetuses and placentae.

No grossly toxic effects were observed in the 24 hr following injection. The levels of radioactivity in all the tissues studied, including the maternal blood, were less than 1% of the injected dose, but Te was shown to have reached the foetuses by days 9 and 10 of gestation (the period of sensitivity for hydrocephalus induction). As foetal age increased, an increasing restriction on Te transport was exercised by the placenta, the mean offspring/maternal blood ratio of radioactivity decreasing from 0·3 on days 8–11 to 0·16 on days 12–21. Autoradiographs of late foetuses showed a marked accumulation of Te in the choroid plexus of the lateral and fourth ventricles and the development of a blood–brain barrier to Te was apparent by day 18. Relatively high Te levels in liver were demonstrated from day 12.

The fact that hydrocephalus is the only foetal abnormality so far associated with Te administration during pregnancy and the present demonstration of its presence in the early embryo in significant quantities suggests that Te may act directly on embryonic rather than on maternal processes. Moreover, the preferential uptake of Te by the developing choroid plexus, which plays a crucial role in the elaboration and reabsorption of cerebrospinal fluid, suggests the interesting possibility that Te may interfere directly with choroid-plexus function.

2595. A puff of aldehyde

Egle, J. L., Jr. (1972). Retention of inhaled acetaldehyde in the dog. *Archs envir. Hlth* **24,** 354.

Egle, J. L., Jr. (1972). Retention of inhaled formaldehyde, propionaldehyde, and acrolein in the dog. *Archs Path.* **94,** 119.

Egle, J. L., Jr. (1972). Effects of inhaled acetaldehyde and propionaldehyde on blood pressure and heart rate. *Toxic. appl. Pharmac.* **23,** 131.

Aldehydes are important constituents of tobacco smoke. We have seen in a previous paper by the author cited above that, in man, the retention of acetaldehyde is independent of the depth of inhalation but may vary from 45–70% with increasing duration of ventilation (*Cited in F.C.T.* 1971, **9,** 596). Since the concentration of acetaldehyde in tobacco smoke may approach 81 μg/40-ml puff, acetaldehyde retention may have a bearing on the development of cardiovascular disease (as the compound is a sympathomimetic) and on alterations in brain metabolism in smokers. A need for further investigation was therefore indicated.

In the first paper cited, the author reports that, in dogs exposed to 0·4–0·8 μg acetaldehyde/ml, total respiratory-tract retention varied from 61% for respiratory rates of 7–9/min to

44% for rates of 44–70/min. These figures are very close to those already reported for the comparable study in man (*Cited in F.C.T.* 1971, **9,** 596). Uptake of acetaldehyde was greater in the upper than in the lower respiratory tract, and was unrelated to tidal volume (within limits of 100–240 ml) or to inhaled concentration (tested over a range of 0·1–3·00 μg/ml).

The second paper reports a parallel study showing that the total respiratory-tract uptake of formaldehyde inhaled by dogs in concentrations in the range of 0·15–0·35 μg/ml was nearly complete and was considerably higher than the uptake of propionaldehyde (tested in the 0·4–0·6 μg/ml range) or acrolein (also tested at 0·4–0·6 μg/ml). The uptake of formaldehyde was close to 100% regardless of concentration, respiratory rate or tidal volume. Retention of acrolein was around 80% regardless of the ventilatory rate or concentration, but showed some decline (from 86 to 77%) as tidal volume increased from 100 to 160 ml. Total-tract retention of propionaldehyde was also in the 75–80% range, but this varied inversely with the ventilatory rate. Propionaldehyde retention was greater than that of acrolein in the lower respiratory tract and lower in the upper tract.

The third paper describes how inhalation of 0·5–30 μg acetaldehyde/ml or 3–200 μg propionaldehyde/ml for 1-min periods affected the blood pressure and heart rate of anaesthetized rats. With acetaldehyde in concentrations of 3 μg/ml or more and with slightly higher concentrations of propionaldehyde, blood pressure rose significantly, falling back again to normal levels some 5 sec after exposure ceased. With acetaldehyde in concentrations of 12–25 μg/ml, the heart rate was accelerated, and slightly higher levels of propionaldehyde again had a similar effect. These findings indicate that higher concentrations of acetaldehyde and propionaldehyde than occur in cigarette smoke would probably be required to affect blood pressure and heart rate in man.

2596. Toxicity profile of 2-chloroethyl isocyanate

Hofmann, A. u. Neufelder, M. (1972). Tierexperimentelle Untersuchungen zur gewerbetoxikologischen Beurteilung von 2-Chloräthylisocyanat. *Arch. Tox.* **29,** 73.

The oral LD_{50} of 2-chloroethyl isocyanate was found to be 396 mg/kg in male rats and 630 mg/kg in female mice. Lethal doses caused death within 1–3 hr, and this was preceded by abrupt liberation of much stomach gas. Survivors suffered hair loss over the next 5 days, and at the end of the 7-day observation period autopsy revealed marked irritation and ridging of the stomach wall.

When dropped into the rabbit eye, 5 μl of this isocyanate caused severe conjunctival irritation and diffuse superficial keratitis. Corneal transparency was not restored for 14 days or more. Application to the skin in rabbits induced redness and mild swelling, and histological examination showed marked inflammatory changes and oedema of the dermis. Inhalation provoked no systemic toxicity but caused irritation of the respiratory tract. In rats, the inhalation LC_{50} of 2-chloroethyl isocyanate was 6·3 ppm with a 6-hr exposure period. Rats exposed to 3·1–3·8 ppm for 10 days survived, but on examination 14 days later showed tracheobronchitis and bronchopneumonia, although no lesions were found elsewhere in the body. In view of these effects, a threshold limit value of 0·02 ppm 2-chloroethyl isocyanate is suggested.

2597. A carcinogenicity test on formaldehyde

Rosenkranz, H. S. (1972). Formaldehyde as a possible carcinogen. *Bull. env. contam. & Toxicol. (U.S.)* **8,** 242.

Formaldehyde is widely used as a germicide, fungicide, insecticide and preservative and is an important intermediate in many industrial processes. Studies of its metabolic fate in mammals have demonstrated a generally rapid conversion to formic acid and subsequent oxidation to carbon dioxide (*Cited in F.C.T.* 1966, **4,** 99) and it has been shown to be a potent mutagen for Drosophila (*ibid* 1966, **4,** 99) and to produce local sarcomas in rats when administered sc (Grasso & Golberg, *Fd Cosmet. Toxicol.* 1966, **4,** 297) although this cannot of course be taken as evidence of carcinogenic activity.

The mutagenic activity of formaldehyde in Drosophila has led, however, to a further investigation of its potential carcinogenicity. The procedure used, an *in vitro* system, was based on the assumption that the activity of chemical oncogens is derived from their ability to alter the DNA of living cells. DNA damage is not readily demonstrated *in vitro* because normal cells are able to repair the damage. The use of cells lacking a normal capacity for efficient DNA repair overcomes this problem to some extent and such cells have been shown to be more sensitive to agents that interact with cellular DNA, such as radiations, radiomimetic agents and carcinogens, than are their normal counterparts.

The system used in the experiment now under consideration consisted of a parent strain of *Escherichia coli* and its DNA polymerase-deficient mutant. The organisms were grown as a confluent culture spread on agar plates, and sterile discs impregnated with formaldehyde or with a suitable control substance were placed on the agar. After incubation of the plates at 37°C for 7 hr the diameters of the zones of inhibition were measured. The five controls used were two carcinogens, methylmethane sulphonate and *N*-hydroxyaminofluorene, and the antibiotics, streptomycin, ampicillin and cycloserine. The diameters (in mm) of the zones of inhibition produced in cultures of the parent strain by formaldehyde and the five control substances (in the order given above) were 59, 42, 0, 26, 28 and 62, while with the mutant strain the corresponding figures were 62, 56, 12, 26, 28 and 62. The authors concluded that formaldehyde damaged the DNA and therefore had carcinogenic potential.

[Undoubtedly formaldehyde appeared in this study to be more toxic to the mutant than to the parent strain and in this respect it resembled the carcinogens tested. The latter, however, showed a much greater relative toxicity, with differences between the inhibition-zone diameters of the two strains of 12–14 mm, compared with a difference of only 3 mm in the case of formaldehyde. Even if one assumes the system to be infallible, this small difference hardly warrants the conclusion that formaldehyde is a potential carcinogen. One would like to see a much more extensive series of cytotoxic agents used in an attempt to determine whether such a small difference can be regarded as having any real meaning.]

2598. Sensitivity to formaldehyde

Logan, W. S. & Perry, H. O. (1972). Cast dermatitis due to formaldehyde sensitivity. *Archs Derm.* **106,** 717.

Some cases of dermatitis have been attributed to contact with free formaldehyde present in melamine-, urea- and phenol-formaldehyde resins (*Cited in F.C.T.* 1963, **1,** 300). This problem has arisen particularly in connexion with resin-treated textiles (*ibid* 1967, **5,** 589), for while the free formaldehyde left after the curing of the resin can easily be removed by washing, there appears to be some possibility that further formaldehyde may be liberated from the finish during contact with the wearer's skin.

A series of cases of allergic contact dermatitis has now been reported in patients receiving orthopaedic treatment involving the use of plaster casts. In all three cases the casts were not

of the usual gypsum type, but were specially strengthened by the incorporation of melamine-formaldehyde resin. The skin reaction was generally apparent within about 1 wk of the application of the cast and was attributed to free formaldehyde, which has been demonstrated in very low concentrations (0·01–0·03%) in the cast material. The patients reacted positively to patch tests with formaldehyde and to 10-day-old resin-strengthened plaster but not to freshly-prepared plaster, thus supporting the diagnosis of contact dermatitis rather than a non-specific irritation associated with the wearing of the cast.

The authors of this paper stress that most plaster casts do not contain any formaldehyde. Use of the resin does, however, impart added strength without increasing the weight of the cast.

2599. Mineral-oil mists in the pressroom

Pasternack, B. & Ehrlich, Laurel (1972). Occupational exposure to an oil mist atmosphere. A 12-year mortality study. *Archs envir. Hlth* **25,** 286.

The suspected hazards of chronic inhalation of mineral oil mists include carcinomas of the stomach and respiratory tract (*Cited in F.C.T.* 1971, **9,** 600), and in the printing industry the main area in which significant exposure to such mists occurs in the pressroom (*ibid* 1972, **10,** 434).

The study now reported was carried out over a 12-yr period among men involved in the production of a newspaper. The pressroom men were exposed to an oil-mist atmosphere, but the compositors, who otherwise formed a comparable group of workers, were not. There were 778 pressroom men, accounting for a total of 5841 man-years of exposure, and 1207 compositors accounting for 9189 man-years. The death rates found in those pressroom men who had started work in this plant when aged 40 yr or more and had been employed in the plant for at least 20 yr were significantly higher than those in either the other pressroom men first employed at a younger age or the compositors. The reasons for this difference in mortality are not clear. No significant differences in death rates were demonstrated between pressroom men and compositors first employed when less than 40 yr old, so that the data recorded for pressmen who had spent most of their working life at this particular plant did not suggest the existence of an occupational mortality hazard. The higher mortality in those taken on to the payroll at 40 yr of age or more, who had presumably worked previously in other plants, suggested that previous employment, as well as smoking habits and personal medical histories, may have been a factor in the findings of this study.

2600. Irritants and intraocular pressure

Ballantyne, B., Gazzard, M. F. & Swanston, D. W. (1972). Effects of solvents and irritants on intraocular tension in the rabbit. *J. Physiol., Lond.* **226,** 12*P*.

Splash contamination of the eyes may be more hazardous in persons suffering from established or incipient glaucoma than in healthy persons, and this paper offers a basis for assessing the possible hazard posed by compounds that may come in contact with the eye.

Intraocular tension was measured in adult female rabbits before and 10 and 60 min after application of various test materials dissolved in saline or polyethylene glycol 300 (PEG). Like propylene glycol and glyceryl triacetate, these solvent vehicles have no irritant effect on the eye and cause no changes in intraocular tension.

Ammonia, butyl carbitol, ω-chloroacetophenone and *o*-chlorobenzylidene malononitrile

each produced a significant rise in intraocular tension at 10 min, followed by a return to near-normal tension by 1 hr. The rise in tension was dependent on the concentration of the toxic agent in water or PEG. Ammonia and chloroacetophenone (up to 0·5%, w/v) induced relatively small rises in intraocular tension, but the rises increased sharply with higher concentrations. Low concentrations of butyl carbitol and chlorobenzylidene malononitrile had less effect than similar concentrations of the other two compounds, and at higher concentrations these compounds produced a more gradual increase in tension. A linear relationship between the volume of solution applied and the percentage change in intraocular tension was observed for 10% (v/v) butyl carbitol in water.

NATURAL PRODUCTS

2601. Betel effects in baboons

Hamner, J. E., III (1972). Betel quid inducement of epithelial atypia in the buccal mucosa of baboons. *Cancer, N.Y.* **30,** 1001.

The chewing of betel quids has been suspected as a major factor in a high incidence of cancer of the mouth in certain areas of the Orient. The principal alkaloid in the betel nut, arecoline, and its metabolite, arecaidine, are alkylating agents and may therefore play some role in any carcinogenic activity the betel nut may possess (*Cited in F.C.T.* 1970, **8,** 114). Some evidence in support of the suggested carcinogenic effect of betal chewing was obtained in a study in which dimethylsulphoxide extracts of betel painted on the cheek pouch of hamsters produced squamous cell carcinomas (*ibid* 1971, **9,** 919).

The buccal mucosa of the baboon closely resembles that of man, and in a study now reported this species was used to investigate the effects of a common type of betel quid (containing betel leaf, areca nut and lime) and of this substance mixed with Maharashtran tobacco. Twelve baboons were fed on a protein-deficient diet (7·2% protein compared with the normal level of 27%) in an effort to emulate the dietary standards common among Indian betel chewers. When the animals had become adapted to this diet, a pouch was fashioned surgically in the buccal mucosa for administration of the materials. The quids (3·5 g) were inserted three times each week, five baboons receiving the basic quids and seven the betel-tobacco mixture. Biopsies were taken from all the pouches after 1, 6, 9, 12, 16, 23, 29, 34 and 42 months, and the opposite buccal mucosa of each animal served as a control.

Histological examination of the biopsies showed chronic inflammatory changes with some areas of epithelial atrophy and others of hyperplasia during the first 6 months. After 12 months the appearance of the epithelium became atypical, particularly in the animals given the betel-tobacco mixture. There was a marked variation in nuclear size and shape in the epithelial cells and loss of cellular polarity. The pathological changes became more severe as the experiment progressed. Ulcerative haemorrhagic lesions occurred in the buccal mucosa of all animals after 30 months. While the betel quid alone had not produced frank malignant change in the buccal mucosa of the baboon after 42 months of treatment, the changes found in animals treated with the betel-tobacco mixture were potentially carcinogenic and it was postulated that the caustic effect of the lime damaged the mucosal tissue, thus making it more susceptible to the weakly carcinogenic effect of the tobacco. It was anticipated that continuation of the treatment would lead to definite malignant invasion.

2602. Galactose toxicity to the chick brain

Knull, H. R., Wells, W. W. & Kozak, L. P. (1972). Galactose toxicity in the chick: Hyperosmolality or depressed brain energy reserves? *Science, N.Y.* **176,** 815.

We have reported (*Cited in F.C.T.* 1971, **9,** 602) that high levels of galactose can interfere with the cellular growth of the developing brain, at least in rats, but no mechanism was proposed to account for this effect. Since then, a paper by Malone *et al.* (*Science, N.Y.* 1971, **174,** 952) has pointed out that a marked hyperosmolality resulted from giving chicks a high concentration of galactose in the drinking water and has suggested that the toxic effects attributed to high levels of galactose in the serum could in fact be due solely to severe hyperosmolar dehydration.

The results of the chick study cited above have now discounted this hypothesis, and the authors propose that the galactose interferes with the supply of glucose to the brain, thus disrupting energy metabolism in this organ. This view is supported by previous indications that dietary galactose depresses levels of glucose in the brain (Kozak & Wells, *Archs Biochem. Biophys.* 1969, **135,** 371).

Two-day-old chicks were put on a semi-synthetic diet containing 40% D-galactose for 2 days, after which some were injected ip with 1 M-D-glucose or 0·5 M-sodium chloride. The convulsions and tremors associated with the galactose treatment ceased in the chicks given a glucose injection and the duration of physical recovery correlated with the raised level of glucose in the plasma. During the recovery phase, brain concentrations of ATP, glucose, fructose 1,6-diphosphate and lactose returned to approximately normal values. Hyperosmolality was shown to accompany hypergalactosaemia but appeared to be a non-specific factor in the toxic effect.

2603. Mellein and hydroxymellein in culture

Moore, J. H., Davis, N. D. & Diener, U. L. (1972). Mellein and 4-hydroxymellein production by *Aspergillus ochraceus* Wilhelm. *Appl. Microbiol.* **23,** 1067.

We mentioned recently (*Cited in F.C.T.* 1972, **10,** 732) the isolation from *Aspergillus ochraceus* cultures of two isocoumarin derivatives that were thought to be possible precursors of ochratoxin. The two compounds were identified as 3,4-dihydro-8-hydroxy-3-methylisocoumarin (mellein) and 3,4-dihydro-4,8-dihydroxy-3-methylisocoumarin (hydroxymellein).

The same group has continued its study of these compounds and has now reported on their production by *A. ochraceus* Wilhelm on various synthetic media and solid substrates. Among the optimal requirements for production on a synthetic medium were concentrations of 80 g sucrose/litre and 6 g L-glutamic acid/litre, zinc and molybdenum supplements for stationary cultures (although they were not required for shaken cultures) and incubation for 10–14 days at 25–30°C. Four natural substrates were evaluated. A trace of mellein and about 5610 μg hydroxymellein/50 g were produced on yellow corn, but neither compound was produced on wheat, peanuts or soya beans. In contrast, ochratoxin was produced on all these substrates in yields of 6500, 4700, 1830 and 1760 μg/50 g on corn, wheat, peanuts and soya beans respectively.

2604. Another tremorgenic toxin

Cole, R. J., Kirksey, J. W., Moore, J. H., Blankenship, B. R., Diener, U. L. & Davis, N. D. (1972). Tremorgenic toxin from *Penicillium verruculosum*. *Appl. Microbiol.* **24,** 248.

Tremor may be a relatively uncommon manifestation of toxic action (*Cited in F.C.T.* 1969, **7**, 402), but the number of fungi found to produce a metabolite evoking a severe tremorgenic response in experimental animals seems to be increasing steadily.

The latest addition is a mycotoxin produced by *Penicillium verruculosum* Peyronel, which has been isolated from peanuts. The chemical structure of the toxin, designated "verruculogen", has not yet been established, but it appears to be more closely related to two tremorgens isolated from a strain of *Aspergillus fumigatus* (Yamazaki *et al. Chem. pharm. Bull., Tokyo*, 1971, **19**, 1739) than to others already isolated from *A. flavus* and from various Penicillium species. Administered orally or ip to mice or day-old cockerels, verruculogen rapidly caused severe tremors, which lasted for several hours in survivors or led to death in tetanic convulsion within about 45 min. Other toxic signs were hypersensitivity to sound and ataxia, accompanied in cockerels by recumbency, head extension and leg-tendon contracture. In both species, tremors were produced by ip doses of about 0·3 mg/kg, while the effective oral doses were slightly above 10 mg/kg in cockerels and 20 mg/kg in mice. The LD_{50} values in cockerels and mice, respectively, were 15·2 and 2·4 mg/kg by the ip route and 365·5 and 126·7 mg/kg for oral administration. Animals given sublethal doses recovered completely in 24–48 hr.

2605. More comment on smoking during pregnancy

Yerushalmy, J. (1973). Congenital heart disease and maternal smoking habits. *Nature, Lond.* **242**, 262.

Fedrick, J., Alberman, E. D. & Goldstein, H. (1973). Reply to Professor Yerushalmy's letter on congenital heart disease. *Nature, Lond.* **242**, 263.

Our recent review of some of the literature dealing with the possible effects of maternal smoking on the foetus mentioned a report suggesting a correlation between congenital heart disease and smoking during pregnancy (*Cited in F.C.T.* 1973, **11**, 673). This allegation has now been countered in the first letter cited above, the author of which failed to find any such association between the incidence of the disease and maternal smoking in a prospective study in which the data on smoking were recorded early in each pregnancy.

The incidence of congenital heart disease among infants born to smokers was found to be very close to that reported by Fedrick *et al.* (*Cited in F.C.T., loc. cit.*) but in the Yerushalmy study the incidence in the offspring of non-smokers was of the same order, rather than considerably lower as was found in the previous study.

Fedrick *et al.* (second paper cited above) offer no explanation for this discrepancy, but mention an additional series of 7000 perinatal deaths in which the difference in the incidence of congenital heart disease between smoking and non-smoking mothers supported the finding in their earlier study. They do, however, strongly refute the other statement by Yerushalmy (*loc. cit.*), repeating his earlier doubts (*Cited in F.C.T.* 1973, **11**, 671) about the effect of maternal smoking on perinatal mortality.

COSMETICS, TOILETRIES AND HOUSEHOLD PRODUCTS

2606. Photosensitization to chlorophenylphenols

Adams, R. M. (1972). Photoallergic contact dermatitis to chloro-2-phenylphenol. *Archs Derm.* **106**, 711.

Bithionol, the halogenated salicylanilides, hexachlorophene and dichlorophene have all been implicated in cases of photosensitization (*Cited in F.C.T.* 1969, **7,** 670), although considering their wide use as germicides at various times the incidence may be considered to be relatively low. A more striking incidence has now been reported with another phenolic material, a mixture of 4-chloro- and 6-chloro-2-phenylphenols, used as a germicide in a liquid soap.

Four workers among a relatively small population of employees and students of a junior college district developed severe photosensitive dermatitis, particularly of the hands and forearms. The condition, which subsided during periods away from their place of employment, was eventually traced to the chlorophenylphenol mixture, which was present in the liquid soap used in that district at a level of 1·8%.

There have apparently been no previous reports of sensitization to this material, but it is considered to be a skin irritant, particularly at concentrations above 2%. Its incorporation in a hand soap is therefore ascribed to ignorance on the part of the soap manufacturer. The author suggests that the absence of reports of sensitization to the chlorophenylphenols and closely related compounds may be partly explained by the fact that they seldom contact the skin in the course of their usual industrial applications.

In patch tests with the individual compounds, all four of the patients described reacted positively to 4-chloro-2-phenylphenol, but only one reacted to the 6-chloro- isomer. Tests with *o*-phenylphenol and with hexachlorophene and other more commonly used germicides were negative in all cases.

2607. The incidence of lanolin allergy

Epstein, E. (1972). The detection of lanolin allergy. *Archs Derm.* **106,** 678.

The alleged infrequency of lanolin allergy has been questioned for some time by European workers (*Cited in F.C.T.* 1967, **5,** 130; *ibid* 1968, **6,** 815), who have found that this allergy often remains undetected when patch tests are carried out with plain lanolin, while lanolin fractions will give a positive result. American investigators believe lanolin allergy to be rare, although whether this is due to a genuinely lower incidence of the allergy in the USA or to some failure in detection has not been investigated until now.

Previous work (*ibid* 1970, **8,** 474) showed that the allergens were to be found mainly in the wool-alcohols fraction of lanolin. In the present study, a preparation of 30% wool alcohols in petrolatum was used for the routine screening of 298 patients in a suburban dermatological practice over a 3-yr period. In view of the problems associated with the correct assessment of a response, patients showing a positive response to 30% wool alcohols were retested with 10% wool alcohols and other lanolin-containing products. Of ten patients who reacted to the initial patch test, five were regarded as false positives on subsequent testing. The remaining five allergic patients had all recently been using ointments or lotions containing lanolin, but in four cases lanolin was one of several allergens used and the extent to which it had contributed to the dermatitis could not be established. However, the fifth patient was allergic to lanolin alone, and termination of its use resulted in significant relief.

Some irritant false-positives are produced by 30% wool alcohols, but the five patients who reacted to 10% wool alcohols were allergic to lanolin. However, four of the five failed to react to pure lanolin, so that their allergy would have been overlooked in patch tests using unmodified lanolin. The numbers involved in this study were too small to show convincingly

that lower concentrations of wool alcohols provide a conclusive test for lanolin allergy, although other work (*ibid* 1969, **7**, 703) suggests that this may be so.

The author of this work concludes that lanolin allergy, although fairly infrequent, is more common than allergy to preservatives such as parabens, and has an incidence similar to that of formaldehyde allergy. However, while lanolin may provoke allergic reactions relatively infrequently, their diagnosis is particularly important on account of the wide range of products in which lanolin is used.

METHODS FOR ASSESSING TOXICITY

2608. Serum enzyme studies in toxicity testing

Korsrud, G. O., Grice, H. C. & McLaughlan, J. M. (1972). Sensitivity of several serum enzymes in detecting carbon tetrachloride-induced liver damage in rats. *Toxic. appl. Pharmac.* **22**, 474.

Members of the group cited above have already reported a study in which they related the development of detectable changes in serum enzyme levels and isozyme patterns to the appearance of morphological damage in the liver and kidney, following treatment of rats with carbon tetrachloride (CCl_4), mercuric chloride, diethanolamine and thioacetamide (Grice *et al. Fd Cosmet. Toxicol.* 1971, **9**, 847). They found that light- and electron-microscopy were more sensitive indicators of toxic effect than the commonly used serum enzyme activities, obvious morphological damage being induced by dose levels considerably lower than those required to produce detectable changes in serum glutamic–oxalacetic transaminase and lactic dehydrogenase. The study has now been extended to a search for enzymes that would respond quantitatively to varying doses of CCl_4 and would indicate minimal liver damage. Attention was concentrated on enzymes normally found mainly in the liver and occurring only at low levels in the serum and erythrocytes.

The studies were carried out in male rats (80–100 days old) fed a standard commercial diet (in the first experiment) or the same commercial diet or a semi-purified diet (in the second experiment). They were killed 18 hr after receiving a single oral dose of CCl_4 within the range 0·001–2·50 ml/kg. In the first study, activities of serum sorbitol dehydrogenase (SDH), fructose-1-phosphate aldolase, isocitrate dehydrogenase and glutamic–oxalacetic and glutamic–pyruvic transaminases were found to be increased in rats given 0·025 ml CCl_4/kg, but higher doses were required to increase the activities of alcohol dehydrogenase, 6-phosphogluconate dehydrogenase, malic dehydrogenase and lactic dehydrogenase. The activities of several of the enzymes were dose-dependent over a wide dose range. Liver weight and fat content and serum urea also increased with a dose of 0·025 ml/kg or more, while serum arginine showed a dose-related decrease. In the second experiment, the enzyme activities were less sensitive to CCl_4, most enzyme activities showing an increase only with a dose of 0·1 ml/kg. SDH was increased by 0·025 ml CCl_4/kg, but only in rats on the semi-purified diet. However, liver fat was increased in animals given 0·025 ml/kg and liver weight was increased in those given 0·05 ml/kg, while morphological damage was detectable in the liver following a dose of 0·0125 ml/kg, the lowest dose given to rats studied histologically. Increasing liver damage was paralleled by increases in some serum-enzyme activities, but overt liver-cell necrosis was not a prerequisite of detectable changes in enzyme activity.

While serum enzyme studies cannot replace histopathological studies, they offer a convenient screening procedure for use in toxicity tests prior to the stage at which the animals can be killed. For this purpose, the present study suggests that SDH and possibly fructose-1-phosphate aldolase hold some promise as indicators of minimal liver damage. SDH proved a particularly sensitive indicator of CCl_4-induced liver damage and there are indications that it may provide an equally sensitive reflection of liver damage due to thioacetamide and dimethylnitrosamine. While it is unlikely that a single enzyme could be used for detecting specific organ damage in toxicity tests, the authors suggest that extensive studies could identify groups of enzymes suitable for detecting various types of liver injury.

2609. Assay systems for microbial toxins

de Waart, J., van Aken, F. & Pouw, H. (1972). Detection of orally toxic microbial metabolites in foods with bioassay systems. *Zentbl. Bakt. ParasitKde Abt. I. Orig.* **222,** 96.

The sensitivity of a wide variety of biological tests has been examined in the hope of finding a rapid and effective system for detecting bacterial and fungal toxins in foods or animal feeds. Three botulinum toxins, two toxins produced by *Escherichia coli*, two staphyloenterotoxins, two aflatoxins and histamine were tested by a total of 32 biological assay systems, involving the use of fish, chick embryos, insects, arthropods, bull spermatozoa, various cell lines, protozoa and several micro-organisms. None of the test systems used were significantly sensitive to all the toxins tested. Notably, however, several protozoa, some fish and the arthropods (Daphnia and Artemia) were sensitive to 0·1% histamine (0·01% in the case of Daphnia), and the arthropods and some fish species reacted significantly to low concentrations of aflatoxin B_1.

[The interest of this paper to the toxicologist lies not in its search for the illusory 'universal' bioassay system, but rather in its demonstration of species possibly appropriate for the detection of individual toxins.]

TOXICOLOGY

2610. Maternal malnutrition and foetal development

McLeod, Katrine I., Goldrick, R. B. & Whyte, H. M. (1972). The effect of maternal malnutrition on the progeny in the rat. Studies on growth, body composition and organ cellularity in first and second generation progeny. *Aust. J. exp. Biol. med. Sci.* **50,** 435.

Smart, J. L., Adlard, B. P. F. & Dobbing, J. (1972). Effect of maternal undernutrition and other factors on birth weight in the rat. *Biologia Neonat.* **20,** 236.

These two interesting studies are concerned with the effects that maternal malnutrition may have on the outcome of pregnancy in the rat.

The authors of the first paper fed three nutritionally inadequate diets to female rats for 2 wk before and then throughout pregnancy. Four groups of rats were studied; the first (control) group was fed a nutritionally adequate diet, the second a protein-deficient diet (6% protein as opposed to 18%, but made isocaloric with the control diet by addition of extra sucrose), the third a calorie-deficient diet (approximately 50% of the control-diet calories,

the bulk being made up with cellulose) and the fourth a diet deficient in both protein (33% of control level) and calories (50% of control). The number, birth weight and mortality of the progeny were recorded and their subsequent growth was studied. The dams were fed the control diet *ad lib.* after parturition and the pups were fed similarly after weaning.

Maternal protein deficiency caused a decrease in the number of mated females producing litters at term (73% compared with 100% in the control group) and a decrease in the size of litters (seven/litter compared with ten in controls). The mean birth weight was also lower than in controls (5·0 and 6·8 g, respectively) and there was permanent stunting of growth even though the young were subsequently fed the control diet. In addition, these small rats appeared less vigorous, as their mortality during lactation was 10·5% compared with a control rate of 3·2%. The progeny of this test group also showed a reduction in organ weights directly attributable to a reduction in the numbers of cells, but no changes in the chemical composition of the carcasses (protein, ash, fat and water) were seen in this or any other group.

Calorie deficiency produced similar but less marked effects, and although birth weight was decreased there was little effect on the subsequent growth of the progeny. The combination of calorie and protein deficiency did not greatly enhance the effects associated with protein deficiency alone.

In a second-generation study, the female progeny of protein-deficient dams were found to be less fertile than the controls and produced stunted litters. A further period of protein deprivation during their own gestation enhanced this infertility and increased the mortality of the young during lactation.

In the second study cited above, the effect of reducing the total food intake of pregnant rats to 50% of the normal level, from day 7 of gestation until parturition, was demonstrated. In this case the only significant effect was a reduction of the weight of the young at birth, the mean body weights being some 12% less than those of the control pups.

[The work reported in these two papers has a great deal of bearing on the interpretation of reproduction and teratology studies on various materials. The necessity of administering large amounts of a test substance in the food often gives rise to reductions in food intake either because the diet is thus rendered unpalatable or because of the percentage of nutritionally valueless material which has to be incorporated. These papers show that this can influence two of the parameters commonly measured in reproduction studies, namely the numbers and the body weights of the young produced. Moreover, the second paper mentioned suggests that at least some of the effect is due to deficiency during the period of organogenesis.

However, a number of questions are left unanswered. The degrees of dietary reduction and nutritional deprivation were extreme and it would therefore be desirable to examine the effects of less severe changes. McLeod *et al.* (*Aust. J. exp. Biol. med. Sci.* 1972, **50,** 435) did not establish whether the reductions in the numbers of litters and in litter size were due to losses before or after implantation or even to an effect on ovulation. Termination of pregnancies prior to parturition and examination of the uteri and ovaries to determine resorptions, offspring dead *in utero* and the numbers of corpora lutea could answer some of the outstanding questions.]

Fd Cosmet. Toxicol. Vol. 11, p. 933. Pergamon Press 1973. Printed in Great Britain

MEETING ANNOUNCEMENTS

ANNUAL MEETING OF THE SOCIETY OF TOXICOLOGY

The annual scientific meeting of the Society of Toxicology will be held at the Washington Hilton Hotel in Washington, D.C., on 10–14 March 1974. Anyone interested may attend. Papers will be presented by members of the Society and by non-members, the latter papers being sponsored by a member.

The programme and information on accomodation and registration for the meeting will be sent to all members of the Society and to non-members presenting papers. All others requiring such information should contact the Secretary: Dr. R. A. Scala, Secretary, Society of Toxicology, Esso Research and Engineering Co., Box 45, Linden, NJ 07036, USA.

CZECHOSLOVAK CONFERENCE ON FOOD AND NUTRITION

The Society of Hygienists of the J. E. Purkyně Czechoslovak Medical Society has announced an International Conference on Food and Nutrition Hygiene to be held in Brno, Czechoslovakia, on 15–17 October 1974. The theme of the conference will be "Hygienic Problems of Nutrition and Technological Development" and the programme will include papers on food additives and contaminants, antimetabolites and microbial factors, and on novel sources of food and the health problems associated with new technological processes.

Further information may be obtained from the Conference Secretary, Krajská hygienická stanice "CIDHA 74", Žerotínovo nám. 3/5, 602 00 Brno, ČSSR.

Fd Cosmet. Toxicol. Vol. 11, p. 934. Pergamon Press 1973. 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:*

Dietary feeding studies of methylcellulose and hydroxypropylmethylcellulose in rats and dogs. By Susan B. McCollister, R. J. Kociba and D. D. McCollister.

Malformations produced in mice and rats by oxidized linoleate. By Margaret G. Cutler and R. Schneider.

Subacute toxicity of inorganic tin as influenced by dietary levels of iron and copper. By A. P. de Groot.

Determination of total migration from plastics-packaging materials into edible fats using a ^{14}C-labelled fat simulant. By K. Figge.

Effect of some variables on the migration of additives from plastics into edible fats. By K. Figge and J. Koch.

Prolonged administration of *Penicillium viridicatum* to mice: Preliminary report of carcinogenicity. By G. M. Zwicker, W. W. Carlton and J. Tuite.

Inhibition of muscle aldolase by penicillic acid and patulin *in vitro*. By S. H. Ashoor and F. S. Chu.

Fate and toxicity of orally administered polyethylene polyphosphonates. By M. Anbar, G. W. Newell and G. A. St. John.

Monographs on fragrance raw materials. By D. L. J. Opdyke.

Selenium in relation to dental caries. By D. M. Hadjimarkos. (Review Paper).

Toxicology: Cost/time. By P. J. Gehring, V. K. Rowe and Susan B. McCollister. (Review Paper).

กำหนดส่ง

Contents continued]

Aims and Scope

The Journal provides a wide-ranging service of general articles and abstracts on food and cosmetics toxicology. The journal is primarily intended for the use of food scientists and technologists, manufacturers and administrators who neither read nor have access to the medical, pharmacological or toxicological literature. In addition, the journal will constitute a medium for the publication of reviews and original articles relating to the fields of interest covered by the British Industrial Biological Research Association.

Instructions to Authors

General. Authors from the United Kingdom should send *Original Papers and Reviews* to the Assistant Editor. All other papers and reviews should be sent to the appropriate Regional Editor. All 'Letters to the Editor' should be sent to the Editor and must be signed before they can be considered for publication.

Submission of a paper to the Editor will be held to imply that it reports unpublished original research, that it is not under consideration for publication elsewhere and that if accepted for the Journal, *Food and Cosmetics Toxicology*, it will not be published again, either in English or in any other language without the consent of the Editor.

Forms of Papers Submitted for Publication. Papers should be headed with the title of the paper, the surnames and initials of the authors (female authors may use one given name) and the names and addresses of the institutions where the work was done. A shortened version of the title not exceeding 45 letters and spaces, to serve as a running title, should be supplied.

In general the text should be subdivided as follows: (a) Summary: brief and self-contained, not exceeding 3% of length of paper (b) Introductory paragraphs (c) Experimental (d) Results, presented as concisely as possible (e) Discussion (if any) and general conclusions (f) Acknowledgements and (g) References. Each subdivision should commence on a separate sheet. Manuscripts should be typewritten on *one side of the paper* and *double spaced*. At least *two copies* should be supplied (one original and one, or preferably two, carbon copies). Papers will be accepted in English, French and German.

References. These should be listed on a separate page, in alphabetical order and arranged as follows: author's name and initials, year, title of the journal (abbreviated according to the rules adopted in the *World List of Scientific Periodicals 1963*, 4th Ed., Butterworth & Co. (Publishers) Ltd. London), volume, first page number:

e.g. Hickman, J. R., McLean, D. L. A. & Ley, F. J. (1964). Rat feeding studies on wheat treated with gamma-radiation. I. Reproduction. *Fd Cosmet. Toxicol.* **2**, 15.

References to books should include the author's name followed by initials, year, title of book, edition, appropriate page number, publisher and place of publication:

e.g. Dow, E. & Moruzzi, G. (1958). *The Physiology and Pathology of the Cerebellum*. 1st Ed., p. 422. The University of Minnesota Press, Minneapolis.

The names of all the authors of papers to be cited should be given when reference is first made in the text. In cases where there are more than two authors subsequent citations should give the first-named author followed by the words *et al.*:

e.g. (McLaughlin, Bidstrup & Konstam, 1963); (McLaughlin *et al.* 1963).

Where more than one paper by the same author(s) has appeared in any one year, the references should be distinguished in the text and the bibliography by the letters, a, b etc. following the citation of the year:

e.g. 1943a, 1943b or (1943a, b).

Footnotes. These as distinct from literature references should be avoided as far as possible. Where they are essential, reference is made by the symbols * † ‡ § || ¶ in that order.

Illustrations and Diagrams. These should be kept to a *minimum* and they should be numbered and marked on the back with the author's name. Legends accompanying illustrations should be typewritten on *separate* sheets. Diagrams and graphs must be drawn in Indian ink on good quality paper or tracing linen. The following standard symbols should be used on line drawings since they are easily available to the printers:

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Tables and Figures. These should be constructed so as to be intelligible without reference to the text, each table and column being provided with a heading. The same information should not be reproduced in both tables and figures.

Chemical Nomenclature. The fundamental principles of organic and inorganic chemical nomenclature are laid down in the I.U.P.A.C. 1957 Rules (Butterworths Scientific Publications, London, 1958, 1959). These are given in *Handbook for Chemical Society Authors* (1961), pp. 16–163.

Other Nomenclature, Symbols and Abbreviations. In general, authors should follow the recommendations published in the *Handbook for Chemical Society Authors* (1961), p. 164 and in the *I.U.P.A.C. Information Bulletin*, No. 13, p. 64, Appendix B (1961). In the title and summary, abbreviations should be avoided; in the Introduction, Results and Discussion they should be used sparingly.

Page Proofs. These will be sent to the first-named author for correction.

Reprints. Fifty reprints of the paper will be supplied free of charge. An order form for additional reprints will accompany the proofs.

Frequency. The Journal will be published bi-monthly.

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