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

†By D. Schmähl. Heidelberg. Federal Republic of Germany.

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Research Section

ONE-YEAR TOXICITY STUDY OF ORANGE G IN THE FERRET

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(*Received 30 March 1977*)

Abstract—Orange G was administered in the diet to groups of five male and five female ferrets, at concentrations providing intakes of 0, 1·5, 15 and 150 mg/kg/day for 12 months. The object of the study was to discover whether the response to Orange G in this species was similar to that found in rodents and pigs. The haemolytic anaemia produced by this colouring in other species was not seen in the ferret, and a no-untoward-effect level of 150 mg/kg/day was established in this test. However, this value cannot be used in estimating an acceptable daily intake of Orange G for man, as it is apparent that the colouring is metabolized differently in the two species.

INTRODUCTION

Orange G (C.I. (1971) no. 16230), also known as Orange GC, D & C Orange No. 3 and Naphthalene Orange Solide GG is disodium 7-hydroxy-8-phenylazonaphthalene-1,3-disulphonate. This colouring is at present permitted for use in food in the UK (The Colouring Matter in Food Regulations 1973, Statutory Instrument 1973, no. 1340). However, a report of the EEC Scientific Committee for Food (Commission of the European Communities, 1975) suggested that Orange G was not toxicologically acceptable for use in food, a suggestion likely to be reflected in future legislation in the EEC countries. A review of toxicological studies so far completed on this material is to be found in the report of a short-term study of its toxicity in pigs (Gaunt, Atkinson & Hardy, 1978).

This paper describes a 12-month feeding study using ferrets. It was carried out to investigate the effects of the colouring in a non-rodent species, and as part of a study of the value of the ferret in safety evaluation.

EXPERIMENTAL

Materials. Orange G was supplied through the Food Colours Committee of the chemical Industries Association. It complied with the following specification of the British Standards Institution (1963): Dye content min. 85%; matter volatile at 135°C†, max 10%; matter insoluble in water†, max 0·1%; matter soluble in di-isopropyl ether†, max 0·2%; subsidiary dyes, max 1%; primary aromatic amine (as aniline), max 0·02%; chloride and sulphate (as sodium salts)†, max 5%; copper†, max 10 ppm; arsenic†, max 1 ppm; lead†, max 10 ppm; heavy metals (as sulphides)†, producing a colour no more intense than that of the reference standard.

Animals. Ferrets, both albino and polecat varieties and approximately 1 yr old, were purchased from a single source and caged in individual galvanized cages with sawdust as bedding on solid floors.

Diet. The animals received a diet consisting of equal weights of ground Spratt's Laboratory Small Animal Diet, Spratt's ZF6 (canned meat), milk and water. This diet was provided for each animal at a rate of 250 g/animal/day, and water was given *ad lib.*

Experimental design and conduct. Groups of five male and five female ferrets were fed diets containing Orange G at concentrations designed to provide intakes of approximately 0, 1·5, 15 and 150 mg/kg body weight/day. The animals were weighed once each week throughout the study and the food consumption was measured over the 24 hr preceding the day of weighing.

Samples of blood were collected from a caudal vein of all animals at 3 and 6 months. Urine was collected during the final week of treatment from all animals from the control and highest-dose groups. The renal concentrating ability was studied from the control and highest-dose groups. The renal concentrating ability was studied by measuring the volume and specific gravity of the urine produced over night [17.00–09.00 hr], following an 8-hr period of water deprivation. Urine produced in the first 5 hr [17.00–22.00 hr] was collected and measured separately. Diluting ability was investigated by measuring the volume and specific gravity of the urine produced over 2 hr following a 20-ml water load.

After treatment for 1 yr, all surviving animals were killed by exsanguination from the aorta under barbiturate anaesthesia. Blood samples were collected for haematological examination. At autopsy, any macroscopic abnormality was noted and the brain, heart, liver, spleen, kidneys, stomach, small intestine, adrenals, gonads and pituitary were weighed. Samples of these organs and of salivary glands, thyroid, thymus, lymph nodes, pancreas, urinary bladder, lungs, stomach and muscle were preserved in 10% buffered formalin. Paraffin-wax sections of these tissues were stained with haematoxylin and eosin for microscopic examination.

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

All blood samples were examined for haemoglobin concentration and packed cell volume, and counts were made of erythrocytes, erythrocytes containing Heinz bodies, reticulocytes and total leucocytes.

RESULTS

During the study, five of the ferrets died or were killed because of ill-health. One male from the 1·5 mg/kg/day group died after losing weight for several weeks and showing general lethargy. Autopsy revealed that an infection of the middle ear had spread through the bones of the skull to affect the brain. Four females, two from the control group and two from the highest dose group, died between 9 and 10 months after the start of treatment. The two controls lost weight for 4 wk and the conjunctivae were very pale. At autopsy the appearance of the blood suggested an anaemia, but otherwise no changes were seen. Histology failed to reveal any major abnormality in the organs sampled. The two animals from the highest-dose group also showed weight loss and general lethargy prior to death, with some dark discharge from the vagina. Autopsy in both cases revealed a grossly distended uterus filled with pus.

At no time during the experiment did the mean body weights of any treated groups differ significantly from the control values, but the body weights of all animals varied considerably over the 12-month period (Table 1), increasing steadily up to wk 21 and then declining to wk 42, when the values were similar to the initial weights. From wk 42 to the end of the study the body weights again increased. Food intake did not seem to be affected by the inclusion of Orange G even at the highest dietary level. However, like the body weight, the food consumption followed a seasonal pattern with a gradual decline from wk 10 to wk 35 followed by a gradual increase, a pattern reflected in the colouring intake (Table 2).

No statistically significant differences were seen between treated and control animals in the values obtained for the various haematological measurements (Table 3) or in the renal concentration and dilution tests. Neither the weights of the organs recorded at autopsy nor these weights expressed relative to body weight (Table 4) showed any significant or consistent deviation from the corresponding control values.

All the histological findings in the treated ferrets appeared with comparable incidence and severity in the controls. Findings in the liver included haemosiderin deposition, which was observed more frequently in females; additionally, many of the animals showed leucocytes in periportal areas. Common findings in the kidney were cystic glomeruli with eosinophilic contents and, less frequently, dilated tubules. Evidence of haemopoiesis was noted in the spleen of most of the animals and occasionally haemosiderin deposition was seen in this organ. The only other findings of note were cysts seen in the uterus and ovaries of almost all the female ferrets.

DISCUSSION

Apart from the two deaths from unknown cause in the control group, the deaths during this study are considered to have been due to the method used for housing the animals. The practice was to keep animals in individual cages with a tray of sawdust as the floor and it is likely that this resulted in the infections that accounted for three of the deaths in this study. In subsequent ferret studies, all animals have been kept on grid floors and the problem has not recurred.

Cyclical variation of body weight and food intake, such as was seen during this experiment, was first mentioned by Harvey & MacFarlane (1958) and has been seen in other studies in these laboratories (Butterworth, Gaunt & Grasso, 1978). One result of these dramatic changes during a single 12-month period was the necessity for frequent review of the dietary concentration of the test material, to maintain a reasonably constant dosage in relation to body weight.

The major changes consequent upon Orange G administration in the other species investigated (Gaunt, 1973; Gaunt, Kiss, Grasso & Gangolli, 1973; Gaunt, Wright, Grasso & Gangolli, 1971) were the formation of Heinz bodies in the erythrocytes and subsequent haemolytic anaemia due to removal of the affected cells from the circulation. These changes were not seen in the ferret at a statistically significant level.

Table 1. *Mean body weights of ferrets fed diets containing Orange G at levels to provide doses of approximately 0–150 mg/kg/day for 1 yr*

Nominal dose (mg/kg/day)	Body weight (kg) at wk										
	0*	5	10	15	20	25	30	35	40	45	50
Males											
0	1·37	1·60	1·73	1·89	1·98	1·93	1·82	1·52	1·33	1·34	1·46
1·5	1·32	1·56	1·62	1·68	1·81	1·88	1·84	1·71	1·41	1·41	1·44
15	1·31	1·54	1·61	1·79	1·92	1·96	1·93	1·73	1·49	1·43	1·56
150	1·30	1·51	1·60	1·67	1·83	1·92	1·73	1·55	1·34	1·27	1·40
Females											
0	0·87	1·00	1·00	1·04	1·05	1·06	1·02	0·81	0·79	0·76	0·80
1·5	0·76	0·84	0·87	0·86	0·88	0·82	0·89	0·75	0·73	0·71	0·70
15	0·79	0·89	0·91	0·89	0·88	0·86	0·88	0·69	0·68	0·67	0·69
150	0·76	0·84	0·90	0·87	0·89	0·85	0·89	0·79	0·74	0·69	0·79

*First day of feeding.

The values for the treated animals no not differ significantly from those of controls ($P > 0·05$ by Lord (1947) range test).

Table 2. *Mean intake of colouring by ferrets fed diets containing Orante G for 1 yr*

Nominal dose (mg/kg/day)	Colouring intake (mg/kg/day)* at wk 0†	10	20	30	40	50	Mean intake (mg/kg/day)
			Males				
1·5	2·2	1·7	1·6	1·7	1·2	1·8	1·6
15	20·9	18·1	16·4	14·3	12·4	19·4	16·7
150	214·8	190·9	142·0	119·7	124·7	187·4	157·2
			Females				
1·5	1·4	1·9	1·9	2·3	1·7	1·9	1·8
15	13·9	18·8	17·4	17·7	18·1	19·3	17·4
150	134·2	223·2	170·6	157·5	150·5	326·1	193·9

*Calculated from body-weight and food-intake values.
†First day of feeding.

Table 3. *Mean haematological findings in ferrets fed diets containing Orange G at levels to provide doses of approximately 0–150 mg/kg/day for 1 yr*

Sex and nominal dose (mg/kg/day)	No. of animals examined	Hb (g/100 ml)	PCV (%)	RBC (10^6/mm^3)	Retics (% RBC)	Heinz Bodies (% of RBC)	WBC (10^3/mm^3)
				Male			
0	5	14·1	48	8·73	0·9	0·8	5·9
1·5	4	15·2	51	8·98	1·2	0·7	7·4
15	5	14·7	49	8·72	1·4	0·7	5·8
150	5	14·9	50	8·96	1·5	1·5	5·3
				Female			
0	3	15·3	49	8·63	1·4	0·5	2·5
1·5	5	14·0	45	7·52	1·4	0·5	1·9
15	5	15·7	48	8·49	1·9	0·7	3·1
150	3	14·2	44	7·74	2·4	1·5	1·8

Hb = Haemoglobin PCV = Packed cell volume RBC = Red blood cells Retics = Reticulocytes
WBC = White blood cells
Values are means for the numbers of animals shown; those for the treated groups do not differ significantly from the control values ($P > 0·05$ by Lord (1947) range test). There was a similar absence of statistically significant differences between treated and control groups in the values for samples taken at wk 12 and 26.

Table 4. *Relative organ weights of ferrets fed diets containing Orange G at levels to provide 0–150 mg/kg/day for 1 yr*

Sex and dose (mg/kg/day)	No. of animals examined	Relative organ weights (g/100 g body weight) Brain	Heart	Liver	Spleen	Kidneys	Stomach	Small intestine	Adrenals	Gonads	Pituitary*	Terminal body weight (kg)
						Male						
0	5	0·47	0·51	2·48	0·86	0·44	0·67	2·18	0·012	0·096	0·52	1·40
1·5	4	0·49	0·54	2·56	0·80	0·51	0·65	2·03	0·012	0·062	0·49	1·37
15	5	0·45	0·51	2·53	0·99	0·46	0·63	2·15	0·012	0·150	0·47	1·47
150	5	0·48	0·51	2·67	1·14	0·45	0·64	2·12	0·012	0·144	0·50	1·26
						Female						
0	3	0·70	0·57	2·68	1·08	0·43	0·77	2·01	0·013	0·024	0·59	0·75
1·5	5	0·76	0·58	2·87	1·06	0·48	0·83	2·45	0·016	0·024	0·82	0·69
15	5	0·77	0·59	2·78	0·95	0·49	0·81	2·46	0·013	0·020	0·67	0·67
100	3	0·70	0·56	2·73	1·08	0·46	0·83	2·44	0·012	0·130	0·40	0·77

*Values for this organ are expressed in mg/100 g body weight.
Values are means for the numbers of animals shown; those for the treated animals do not differ significantly from the control values ($P > 0·05$ by Lord (1947) range test).

The slightly higher count of Heinz bodies and reticulocytes at wk 52 in the animals given 150 mg/kg/day may have reflected a marginal effect similar to that seen in other species, although the values reported were within the control range for the ferret.

The explanation for this lack of response was demonstrated in a metabolism study in which urinary metabolites of Orange G were identified and measured in several different species (Gaunt, 1973). This demonstrated a basic difference between the fer-

ret and the other species studied, including man. The ferret did not cleave the azo linkage in the colouring to make the active aniline moiety available for further metabolism, and the responsibility of this difference in metabolic activity for the apparent insensitivity of the ferret was confirmed in a continuation of the same study, showing that the ferret produced Heinz bodies in response to the oral administration of aniline. Since the ferret differs from man in this metabolic ability, it is clear that it is an unsuitable species for the routine screening of this type of material.

The results of this study show a no-untoward-effect dose in the range of 160–190 mg/kg/day, the mean intakes for males and females, respectively, at the highest dietary level. However, the difference between the metabolism of this colouring in the ferret and in other species including man, prevents the use of this figure in the estimation of an acceptable daily intake for man. This study emphasizes the importance of metabolic studies in any toxicological investigation. It also demonstrates the continuing requirement for investigations into the biological effects of a material in a range of species. The evaluation of the data in this study indicates the importance of studying mechanisms of action, particularly in relation to any differences between species, when an attempt is made to assess the toxicity of a material.

REFERENCES

British Standards Institution (1963). Specification for Orange G for Use in Foodstuffs. BS 3612:1963.

Butterworth, K. R., Gaunt, I. F. & Grasso, P. (1978). A nine-month toxicity study of diethylene glycol monoethyl ether in the ferret. *Toxicology*. In press.

Commission of the European Communities (1975). Report of the Scientific Committee for Food on the Revision of the Directive on Colouring Matters Authorised for Use in Foodstuffs Intended for Human Consumption. Ref. 2635/VI/75-E.

Gaunt, I. F. (1973). Studies on the Relationship between Heinz Bodies and Haemolysis in Laboratory Animals and the Evaluation of Heinz body Production in Toxicological Investigations. Ph. D. Thesis, University of London.

Gaunt, I. F. Atkinson, H. A. C. & Hardy, J. (1978). Long term toxicity of Orange G in mice. *Toxicology*. In press.

Gaunt, I. F., Kiss, I. S., Grasso, P. & Gangolli, S. D. (1973). Short-term toxicity of Orange G in pigs. *Fd Cosmet. Toxicol.* **11**, 367.

Gaunt, I. F., Wright, M., Grasso, P. & Gangolli, S. D. (1971). Short-term toxicity of Orange G in rats. *Fd Cosmet. Toxicol.* **9**, 329.

Harvey, N. E. & Macfarlane, W. V. (1958). The effects of day length on the coat-shedding, body weight and reproduction of the ferret. *Aust. J. biol. Sci.* **2**, 187.

Lord, E. (1947). The use of range in place of standard deviation in the *t*-test. *Biometrika* **34**, 41.

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SHORT-TERM TOXICITY OF FURFURYL MERCAPTAN IN RATS

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(*Received 30 March 1977*)

Abstract—Groups of 15 rats of each sex were given daily doses of 0, 1, 3 or 30 mg furfuryl mercaptan/kg by stomach tube for 13 wk. No effects attributable to treatment were apparent in the analysis of urine or serum, in renal function tests or in the histological examination. At the highest dose level, a decrease in body-weight gain was associated with a reduced food intake, and there were many differences in organ weights. These consisted mainly of reduced absolute weights together with increased relative values and were thought to be associated with the lower body weights. There were some increases in haemoglobin concentration and packed cell volume at the highest dose level, but these were not considered to represent a toxic effect. The results of this study indicate a no-untoward-effect level of 3 mg/kg/day, although in view of the nature of the findings at 30 mg/kg/day, the actual level seems likely to be higher.

INTRODUCTION

Furfuryl mercaptan (2-furylmethanethiol) is a volatile liquid with an odour which, at low concentrations, resembles roasted coffee. It is used as a constituent of flavourings, particularly for use in chocolate, fruit, nuts and coffee.

Currently there are no regulations relating specifically to the use of flavourings in the UK. However, the Food Additives and Contaminants Committee (1976) classified furfuryl mercaptan in Appendix 6, among flavourings regarded as provisionally acceptable for food use. The Committee requested a 90-day study on a sensitive species and recommended a provisional limit in food of 30 ppm, which should not be exceeded on toxicological grounds. The use of furfuryl mercaptan was considered earlier by the Council of Europe (1974) and it was listed among the artificial flavourings that may be added temporarily to foodstuffs without hazard to public health. This report indicated that there was a need for data relating to short-term studies in animals and to the metabolic fate of the compound. In the USA it was given GRAS status by the Flavouring Extract Manufacturers' Association (1965).

There are no published data concerning the safety-in-use or metabolism of this material. However, in general, a proportion of an oral dose of mercaptans is known to be excreted unchanged in the urine, whilst some is converted in the liver by transthiolation to the corresponding alcohol and 'sulphate'. The major route of excretion of 2-furanmethanol, the parent alcohol of furfuryl mercaptan, is thought to be oxidation to 2-furanoic acid, followed by conjugation with glycine and excretion in the urine (Williams, 1959).

The present short-term toxicity study was carried out as part of the BIBRA programme for the safety evaluation of flavouring agents.

EXPERIMENTAL

Materials. The furfuryl mercaptan used was supplied by Bush Boake Allen Ltd., London, and complied with the following specification: a clear, colourless liquid, purity 99% (by gas–liquid chromatography), specific gravity (20°C) 1·128, refractive index (n_D^{20}) 1·529 b.p. 155°C. Corn oil was supplied by J. Sainsbury Ltd., London.

Animals and diet. Weanling rats of a Wistar strain obtained from a specified-pathogen-free colony (A. Tuck & Son, Rayleigh, Essex) were fed Spillers' Laboratory Small Animal Diet and given tap-water *ad lib.* They were housed at 20 ± 1°C with a relative humidity of 50–70%.

Experimental design and conduct. Groups of 15 male rats (body weight 70–80 g) and 15 females (body weight 60–70 g) were given furfuryl mercaptan by oral intubation daily (7 days/wk) at dose levels of 0 (control), 1, 3 or 30 mg/kg for 13 wk. In addition, groups of five rats of each sex, of similar body weight and from the same batch of animals, were given daily doses of 0, 3 or 30 mg furfuryl mercaptan/kg for 2 or 6 wk. For administration the compound was dissolved in corn oil and the concentrations were adjusted so that all rats, including the controls, which were given corn oil alone, received 5 ml/kg/day. The solutions were prepared twice weekly.

The animals were examined daily for abnormalities of condition or behaviour and were weighed initially, on days 1, 6 and 9, and then weekly up to wk 12. Food and water intakes were measured over the 24-hr period preceding the day of weighing.

Urine was collected from all rats during the last days of treatment and examined for appearance, microscopic constituents and content of glucose, ketones, bile salts, protein and blood. A concentration

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test was carried out at the same time. This involved measuring the specific gravity and volume of the urine produced in a 6-hr period of water deprivation and of that produced during the 2-hr period after a water load of 25 ml/kg. In the animals examined at wk 6 and 13, similar measurements were made on the urine produced during the 4-hr period commencing after 16-hr without water.

The animals were killed by exsanguination from the aorta under barbiturate anaesthesia 24 hr after the final dose of furfuryl mercaptan and following an overnight period without food. Samples of the blood obtained were used for haematological examination and, at wk 13, for serum analysis. The animals were examined for macroscopic abnormalities and the brain, heart, liver, spleen, kidneys, stomach, small intestine, caecum, adrenal glands, gonads, pituitary and thyroids were weighed. Samples of these organs and of lung, various lymph nodes, salivary gland, trachea, oesophagus, colon, rectum, spinal cord, urinary bladder, pancreas, skeletal muscle and uterus or prostate and seminal vesicles were preserved in 10% buffered formalin. Paraffin-wax sections of these tissues were stained with haematoxylin and eosin for microscopic examination. Smears of femoral bone marrow were prepared and stained, but in the absence of haematological changes, they were not examined.

The blood was examined for haemoglobin concentration and packed cell volume together with counts of erythrocytes and total leucocytes. Slides were prepared in all cases for the enumeration of the different types of leucocytes and reticulocytes, but these estimations were confined to the rats from the control group and those given 30 mg furfuryl mercaptan/kg. Serum separated from the remainder of the blood was analysed for the content of urea, glucose, total protein and albumin, as well as for the activities of glutamic–oxalacetic transaminase, glutamic–pyruvic transaminase and lactic dehydrogenase.

RESULTS

No differences between test and control animals were seen in the condition or behaviour of the rats at any dose level. Body weights were lower in both male and female rats given 30 mg/kg/day than in the corresponding controls. The differences were significant from days 6–9 onwards and the extent of the difference was similar for both sexes, being approximately 16 and 12% of control values at day 83 in males and females respectively (Table 1). Over the whole experimental period, the mean food comsumption by both sexes given furfuryl mercaptan at 30 mg/kg/day was reduced by 15–20% compared with the intake of the untreated animals. These differences were statistically significant. The differences from controls in the individual weekly values were statistically significant for about half the measurements (Table 1). The overall mean water intake of the same rats was greater than that of the controls but not significantly so. Consideration of the individual weekly data showed that the water intake of these animals was greater than that of the controls between days 6 and 35 and that in the early part of the experiment some of the differences from control values were statistically significant.

Haematology data (Table 2) showed no statistically significant deviations from the control values in the rats given 1 or 3 mg/kg. At the highest level of treatment the haemoglobin concentration at wk 13 was significantly higher than the control value in the male rats and higher, but not significantly so, in the female rats. The packed cell volume was significantly greater than that of the controls in the males of this group after wk 6 and 13, as was the total leucocyte count in the females at wk 6. The comparable values for packed cell volume in the females were higher than the controls but not to a statistically significant degree. The proportion of lymphocytes in the male rats at the highest dose level was less than that of the controls at 2 wk but in females the proportion of these cells was greater. There were no differences in the differential leucocyte count at the later examinations.

There were no significant deviations from control values in the results of the serum analyses or in the results of the renal concentration and dilution tests

Table 1. *Mean values of body weight and food consumption of rats given daily oral doses of 0–30 mg furfuryl mercaptan/kg*

Dose level (mg/kg)	Body weight (g) at day				Food consumption (g/rat/day) at day				Mean food consumption (g/rat/day)
	0†	28	55	83	1	28	55	83	
					Males				
0	74	235	318	376	11·1	17·6	19·1	17·3	16·6
1	75	246	331	387	12·5	19·1	18·9	16·5	16·8
3	73	240	321	364	11.3	18·1	18·1	15·9	16·0
30	76	211**	273***	316***	9·5*	16·7	16·9	14·9*	14·3**
					Females				
0	70	171	211	234	11·0	14·0	17·3	14·9	14·1
1	70	177	221*	240	11·5	14·9	16·1	14·3	13·9
3	68	173	216	238	10·4	14·1	16·5	14·3	13·9
30	70	160*	189***	206**	8·5**	14·1	12·6*	11·3***	11·6***

†First day of treatment.

Figures for body weight are the means for groups of 15 animals and those for food consumption are the means for three cages of five animals. Those marked with asterisks differ significantly (Student's t test for body weight, the ranking test of White (1954) for mean consumption and the Lord (1947) test for individual consumption values) from the values for the controls: $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

Table 2. *Haematological results in rats given daily doses of 0–30 mg furfuryl mercaptan/kg for 2, 6 or 13 wk*

Sex and dose level (mg/kg/day)	Hb (g/100 ml)	PCV (%)	RBC (10^6/mm^3)	Retics (% of RBC)	Leucocytes Total (10^3/mm^3)	Differential (%) N	E	L	M
				Wk 2					
Males									
0	11·9	38	5·66	1·4	5·51	14	0	86	0
3	12·1	39	5·69	—	5·22	—	—	—	—
30	12·2	40	5·98	1·1	5·42	20	0	78*	2
Females									
0	12·3	40	5·84	0·9	4·91	23	0	76	1
3	12·1	40	5·85	—	6·48	—	—	—	—
30	12·7	41	6·07	0·7	6·85	15**	0	84**	0
				Wk 6					
Males									
0	15·9	45	8·37	0·9	5·52	16	0	83	1
3	15·3	44	7·81	—	5·32	—	—	—	—
30	16·3	47*	8·32	0·6	7·53	12	1	86	1
Females									
0	15·5	44	7·80	0·7	4·19	15	0	83	2
3	14·8	42	7·54	—	3·31	—	—	—	—
30	15·6	45	8·29	1·1	6·50*	13	0	86	1
				Wk 13					
Males									
0	16·1	45	8·52	0·9	5·46	22	1	75	2
1	15·8	44	8·14	—	5·21	—	—	—	—
3	15·7	44	8·17	—	5·27	—	—	—	—
30	17·0*	46*	8·61	0·7	5·73	23	0	75	1
Females									
0	15·3	43	7·44	0·6	3·45	18	2	79	1
1	16·3	43	7·25	—	3·99	—	—	—	—
3	15·8	43	7·56	—	4·05	—	—	—	—
30	16·8	44	7·56	0·7	4·06	20	2	78	1

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

Figures are the means for groups of five rats at wk 2 and 6 and groups of 15 at wk 13 and those marked with an asterisk differ significantly (Student's t test) from the appropriate control values: $*P < 0{\cdot}05$; $**P < 0{\cdot}01$.

and urinary cell counts. No abnormal constituents were found in the urine.

The changes in organ weight were confined largely to animals on the highest dose level (30 mg/kg/day), the lower organ weights in this group generally reflecting the lower body weights. Considering the differences at this dose level in more detail, the relative brain weights were increased throughout the study (Table 3), although the only statistically significant difference in absolute weights was a lower value in males at wk 2. The only significant difference in heart weight was a lower value found in males at wk 13, whilst the relative values were higher in males at wk 6 and in females at wk 13. The liver weight was lower in both sexes at wk 2 and in males at wk 13 but the only difference in relative weight was an increased value in females at wk 13. In the case of the spleen, the weight was lower in both sexes at wk 13, in females at wk 6 and in males at wk 2. All of the relative values were similar to those of the control except for a lower value in the males at wk 2. Kidney weights were lower than in controls in the males at wk 2 and 13 and in the females at wk 6. The relative values for this organ were lower than the controls in the males at wk 2 but were increased in males at wk 6 and in both sexes at wk 13. The caecum weight was lower than that of the controls in females at wk 6, whilst the relative values were increased in both sexes at wk 13. There were significantly lower values for small-intestine weight at wk 13 with a higher relative value in the males. There were increases in relative stomach weights and in relative adrenal weights in both sexes at wk 6 and 13, with no differences in the absolute values. The only statistically significant difference in ovary weight was a reduced value at wk 6, whilst in the case of the testis the absolute weights were similar to the controls but the relative values were higher at wk 6 and 13. The only statistically significant difference between these rats and the controls in terms of pituitary weight was a lower value in females at wk 6, and in the case of the thyroid a higher value for the relative weight was apparent in females at wk 13.

In rats given the two lower dose levels, statistically significant differences in organ weight compared with the controls were scattered. There were lower values for the kidney and stomach weights in the females given 3 mg/kg/day at wk 6, although only the kidney weight was reflected as a lower value in the relative figures. The only difference at the lowest dose level was an increased small-intestine weight in the females at wk 13 with no significant difference in the relative values.

The incidence of gross changes at autopsy and of

Table 3. *Relative organ weights of rats given daily doses of 0–30 mg furfuryl mercaptan/kg for 2, 6 or 13 wk*

Sex and dose level (mg/kg/day)	Relative organ weight (g/100 g body weight)												Terminal body weight (g)
	Brain	Heart	Liver	Spleen	Kidneys	Stomach	Small intestine	Caecum	Adrenals†	Gonads‡	Pituitary†	Thyroid†	
						Wk 2							
Male													
0	1·10	0·39	3·62	0·48	0·84	0·66	4·44	0·45	30	1·22	4·2	6·7	154
3	1·14	0·39	3·30	0·45	0·87	0·72	4·39	0·46	28	1·34	4·0	7·5	148
30	1·22*	0·42	3·54	0·37*	0·78*	0·72	4·31	0·45	31	1·31	4·1	7·8	133**
Female													
0	1·24	0·41	3·86	0·42	0·87	0·85	4·46	0·50	39	72	5·6	7·9	128
3	1·20	0·42	3·81	0·53	0·85	0·76	4·68	0·52	42	63	5·4	7·2	128
30	1·29	0·41	3·72	0·38	0·87	0·78	4·45	0·48	41	61	5·4	8·6	116
						Wk 6							
Male													
0	0·66	0·29	2·92	0·21	0·64	0·54	2·67	0·41	19	1·11	2·3	4·6	273
3	0·71	0·32*	2·85	0·24	0·69	0·54	2·73	0·45	26*	1·02	3·0	4·9	253
30	0·79*	0·32**	2·99	0·25	0·73**	0·62*	3·10	0·42	24*	1·34*	2·2	6·1	219*
Female													
0	0·93	0·34	2·92	0·31	0·70	0·64	3·28	0·46	33	60	5·7	5·5	185
3	0·92	0·32	2·66	0·28	0·62**	0·56	3·02	0·38	36	57	5·4	6·2	181
30	1·05*	0·34	3·20	0·31	0·72	0·77**	3·60	0·45	46	59	4·8	6·2	154**
						Wk 13							
Male													
0	0·50	0·27	2·74	0·18	0·57	0·44	2·22	0·31	16	0·91	2·0	5·4	370
1	0·48	0·26	2·67	0·17	0·56	0·42	2·25	0·29	16	0·84	2·2	5·2	382
3	0·49	0·26	2·63	0·18	0·57	0·43	2·33	0·31	17	0·93	2·0	5·1	365
30	0·61***	0·29	2·79	0·18	0·62**	0·56***	2·46**	0·36*	21*	1·10***	2·4	6·0	297***
Female													
0	0·71	0·31	2·47	0·22	0·57	0·54	2·79	0·38	30	46	3·1	6·7	234
1	0·74	0·31	2·57	0·23	0·57	0·56	2·97	0·38	31	46	3·6	7·2	239
3	0·74	0·33	2·71	0·24	0·62	0·55	3·09	0·41	37	58	4·0	6·2	230
30	0·82***	0·34**	2·73**	0·24	0·64***	0·63***	2·95	0·42*	38*	57	4·0	9·6**	195***

†Relative weights for these organs are expressed in mg/100 g body weight.
‡Relative weights for female gonads are expressed in mg/100 g body weight.
Figures are means for groups of five rats at wk 2 and 6 and groups of 15 at wk 13 and those marked with asterisks differ significantly (Student's *t* test) from the appropriate control values: *$P < 0·05$; **$P < 0·01$; ***$P < 0·001$.

histological findings was similar in the control rats and in those given 30 mg furfuryl mercaptan/kg/day. The histological findings consisted of a low incidence of slight peribronchial cuffing with lymphocytes.

DISCUSSION

Treatment of these rats with furfuryl mercaptan at doses up to 30 mg/kg/day did not influence the results of the analyses of the urine and serum or of the renal function tests. With administration of doses up to 3 mg/kg/day, there were no abnormal findings, with the exception of isolated differences in organ weight. These differences were seen only in one sex and generally were not accompanied by changes in the values expressed relative to body weight. It is unlikely, therefore that they were related to treatment, and the level of 3 mg/kg/day can be considered a no-effect level.

The major finding at the highest dose level was a reduction in weight gain, evident from an early stage of the experiment. This was accompanied by a reduced food intake, the overall reduction being 14 and 18% in males and females, respectively. The final body weight was reduced by 12–16%, a reduction similar to the 15–20% reported by Schwartz, Tornaben & Boxill (1973) for rats maintained on a food intake of 87·5% of the *ad lib.* level. Hence it is likely that the failure of the animals to gain weight at a normal rate when given this high dose level was due to the reduced food intake. However, since the dose was given by intubation rather than in the diet, the reduced intake of food could not have been due to an unpalatable diet and is likely to have been the result either of a systemic toxic effect or of a local effect on the gastro-intestinal tract resulting from the administration of the material. There is no evidence from the present study to indicate which of these mechanisms was responsible.

Schwartz *et al.* (1973) reported changes in organ weight and relative organ weight comparable with those seen in the present study. Since the only difference between their various groups of animals was the amount of food provided, their study supports the view, additionally strengthened by the absence of histological abnormalities in furfuryl mercaptan-treated rats, that the organ-weight findings in the currently described study did not reflect a direct effect of furfuryl mercaptan on any of the organs. Schwartz *et al.* (1973) also found higher values for packed cell volume and haemoglobin concentration in rats given reduced quantities of food. It is unlikely, therefore, that the similar changes in the haematological picture found in the present experiment were due to the treatment.

The slight changes in the proportions of the different types of leucocyte were found only at wk 2, and the differences were of opposite direction in the two sexes. This does not suggest an effect of the treatment.

The results of this study have shown that the no-untoward-effect level for furfuryl mercaptan is at least 3 mg/kg/day. At the higher dose (30 mg/kg/day) the only effects possibly attributable to treatment were the reductions in food intake and body-weight gain, the other effects being secondary to the altered body weights. Hence it is likely that the true no-untoward-effect level is considerably higher than 3 mg/kg/day. Nevertheless, with application of the traditional 100-fold safety factor, this study suggests an acceptable daily intake for man of 0·03 mg/kg/day or 2·1 mg/day for a 70-kg adult.

REFERENCES

Council of Europe (1974). Natural Flavouring Substances, Their Sources and Added Artificial Flavouring Substances. Partial Agreement in the Social and Public Health Field. List 2, no. 2202, p. 324. Strasbourg.

Flavouring Extract Manufacturers' Association (1965). Survey of flavouring ingredient usage levels. No. 2493. *Fd Technol., Champaign* **19** (2), part 2, 155.

Food Additives and Contaminants Committee (1976). Report on the Review of Flavourings in Food. FAC/REP/22, Ministry of Agriculture, Fisheries and Food. HMSO, London.

Lord, E. (1947) The use of range in place of standard deviation in the *t*-test. *Biometrika* **34**, 41.

Schwartz, E., Tornaben, J. A. & Boxill, G. C. (1973). The effect of food restriction on hematology, clinical chemistry and pathology in the albino rat. *Toxic. appl. Pharmac.* **25**, 515.

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

Williams, R. T. (1959). *Detoxication Mechanisms. The Metabolism and Detoxication of Drugs, Toxic Substances and Other Organic Compounds.* 2nd Ed., p. 550. Chapman & Hall Ltd., London.

Fd Cosmet. Toxicol. Vol. 15. pp. 389–399. Pergamon Press 1977. Printed in Great Britain

SHORT-TERM TOXICITY STUDY OF DI-(2-ETHYLHEXYL) PHTHALATE IN RATS

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Abstract—Groups of 15 male and 15 female rats were given diet containing 0 (control), 0·2, 1·0 or 2·0% di-(2-ethylhexyl) phthalate (DEHP) for 17 wk. At the two higher treatment levels there was a reduced rate of body-weight gain and food intake. A paired-feeding study showed that the reduced food intake did not account fully for the reduced growth rate. There was a decreased packed cell volume in both sexes given 1 or 2% DEHP, and in the male rats given these levels the haemoglobin concentration was also reduced. However, there was no decrease in the total erythrocyte count. Renal concentrating and diluting ability was reduced in the females receiving 2% DEHP. There were no differences between treated and control rats in the results of the serum analyses. With the exception of the liver weights, which were higher, the absolute weights of most organs in the rats given 1 or 2% DEHP were lower than those of the controls, while the relative values were greater. This pattern was considered to be related to the lower body weights at autopsy. At these treatment levels, the relative testis weight was markedly decreased and histopathological examination revealed severe seminiferous tubular atrophy and cessation of spermatogenesis. The testis weight of the rats fed 0·2% DEHP was not reduced, but histologically there was evidence of decreased spermatogenesis. At wk 17, castration cells were observed in the pituitary at all treatment levels, the incidence being dose-related. There were no other histopathological changes attributable to DEHP treatment. In view of the testicular changes and the increase in liver weight at all treatment levels, it was not possible to establish a no-untoward-effect level for DEHP in this study.

INTRODUCTION

Esters of phthalic acid are used extensively as plasticizers for a wide variety of synthetic polymers and in particular for polyvinyl chloride (PVC). Finished PVC, the applications of which include food-packaging materials, household and vehicle furnishings, medical tubing and blood-storage bags, may contain up to 40% by weight of plasticizer (Autian, 1973). Di-(2-ethylhexyl) phthalate (DEHP), the phthalate ester most widely used in PVC formulations, accounts for approximately 25% of an annual world production of phthalate ester plasticizers totalling at least $1{\cdot}5 \times 10^9$ lb (Fishbein & Albro, 1972).

There are no specific regulations in the UK governing the use of DEHP in packaging materials. In the USA it is permitted for a number of food-contact uses, notably in resinous and polymeric coatings (Sec. 121.2514 of the Code of Federal Regulations), in paper and paperboard for contact with aqueous and fatty foods (Sec. 121.2526) and in closures with sealing gaskets (Sec. 121.2550).

DEHP has a low order of acute toxicity. Oral LD_{50} values of 26–> 34 g/kg for the rat and of 34 g/kg for the rabbit have been reported (Fassett, 1967; Hodge, 1943; Shaffer, Carpenter & Smyth, 1945). Following ip administration, the LD_{50} of DEHP was greater than 23·8 g/kg in the rat (Hodge, 1943). By the same route in mice, LD_{50} values of 14·2 (Calley, Autian & Guess, 1966) and 33·8 g/kg (Lawrence, Malik, Turner, Singh & Autian, 1975) were obtained. There is also a report of a value greater than 128 g/kg (Hodge, 1943). Histological examination revealed cloudy swelling of the liver and kidneys in rats receiving a lethal oral dose of DEHP (Shaffer *et al.* 1945). These authors also reported that an adult human male who ingested a single dose of 5 ml DEHP suffered no ill effects, while another subject who ingested 10 ml suffered mild gastric disturbances and moderate catharsis.

A short-term study involving dietary administration of DEHP to male rats at levels equivalent to 200, 400, 900 and 1900 mg/kg/day established a no-untoward-effect level of 200 mg/kg/day (Shaffer *et al.* 1945). The only effect noted at 400 mg/kg was a slight retardation of growth rate. At the two highest dietary levels, however, "tubular atrophy and degeneration in the testes, resembling senile changes" were observed in all the rats.

Carpenter, Weil & Smyth (1953) and Harris, Hodge, Maynard & Blanchet (1956) have reported 2-yr feeding studies in rats using dietary levels of DEHP providing intakes of 20–250 mg/kg/day. At levels below about 70 mg/kg/day no effects were seen, while the higher levels produced statistically significant increases in relative liver and kidney weights and a decreased rate of body-weight gain. Similar findings were made by Nikonorow, Mazur & Piekacz (1973) in a 1-yr feeding study in rats. No adverse effects were observed in guinea-pigs given DEHP at a dietary level providing an intake of approximately 70 mg/kg/day for 1 yr (Carpenter *et al.* 1953).

Although the studies considered above fostered the

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view that little hazard was associated with the use of DEHP in its specific applications, several more recent findings have caused renewed interest in the toxicology of the phthalate esters. First, the finding of appreciable quantities of phthalic acid in a species of deep-sea jellyfish (Morris, 1970) and of phthalate esters in bovine heart muscle (Nazir, Alcaraz, Bierl, Beroza & Nair, 1971) and in various species of fish (Mayer, Stalling & Johnson, 1972; Stalling, Hogan & Johnson, 1973) has suggested that phthalate esters are more widely distributed in the environment than had previously been thought, and that they are capable of accumulating in biological systems. Secondly, the discovery that phthalate esters can be extracted from PVC by blood and other fluids (Jaeger & Rubin, 1970a,b; Marcel & Noel, 1970; Neergaard, Nielsen, Faurby, Christensen & Nielsen, 1971) has raised the question of toxicity following iv administration. Jaeger & Rubin (1970a) found that blood stored for 21 days in PVC bags contained 50–70 ppm DEHP, so that a transfusion of 14 pints of blood could contribute, under these conditions, an iv dose of up to 350 mg DEHP. Confirmation of this view was provided by the identification of DEHP in the tissues of patients recently transfused with blood stored in PVC bags (Jaeger & Rubin, 1972) and in infants after the use of umbilical catheters (Hillman, Goodwin & Sherman, 1975). According to Jones, Kahn, Groves & Napier (1975) the levels of DEHP found in stored blood are sufficient to cause significant growth inhibition in cultures of human diploid cells.

Another area of concern relates to the possible teratogenic effects of phthalate esters in experimental animals. Singh, Lawrence & Autian (1972) reported that each of eight phthalate esters administered ip to pregnant rats at dose levels of 0·3–10·0 ml/kg on day 5, 10 and 15 of gestation increased the number of dead and resorbed foetuses and decreased the mean weight of surviving foetuses. In contrast to the effects of six phthalate esters of lower molecular weight, DEHP produced no skeletal abnormalities, and gross abnormalities (mainly haemangiomas of the legs) were produced only at a dose level of 10 ml/kg. Singh, Lawrence & Autian (1974) also described mutagenic and antifertility effects in male mice given a single ip injection of DEHP at dose levels of 12–25 g/kg. At the highest dose level, females placed with treated males at intervals over a 12-wk observation period showed decreases in the incidences of pregnancy, numbers of implantations and numbers of offspring and an increased number of early foetal deaths. However, the dose levels used in both of these studies (Singh *et al.* 1972 & 1974) were so high as to render the results of doubtful toxicological significance. In addition the significance of these findings is reduced by the use of the ip route of administration.

Studies in the rat have shown that orally administered DEHP is rapidly eliminated, 42 and 57% of a single dose appearing in urine and faeces, respectively, within 7 days (Daniel & Bratt, 1974). Neither these studies, nor those of Lake, Gangolli, Grasso & Lloyd (1975) have provided any evidence of storage of phthalate residues in the liver. It appears that orally administered DEHP is substantially hydrolysed by non-specific intestinal lipases to mono-(2-ethylhexyl) phthalate (MEHP) (Albro & Thomas, 1973; Rowland, 1974). The subsequent metabolic fate of absorbed MEHP proceeds through the formation and urinary excretion of metabolites derived from the ω and $\omega - 1$ oxidation products of the alkyl moiety of MEHP (Albro, Thomas & Fishbein, 1973; Daniel & Bratt, 1974). Further hydrolysis of MEHP can occur to a limited extent in both the intestine and the liver (Albro & Thomas, 1973), accounting for the presence of some 2–3% of free phthalic acid among the urinary metabolites of DEHP (Albro *et al.* 1973).

In view of the increasing use of phthalate esters, the continuing uncertainty over their safety in use and the lack of a short-term study carried out according to current standards, the present study was performed as part of the BIBRA safety evaluation programme.

EXPERIMENTAL

Materials. DEHP was supplied by BP Chemicals International Ltd., Epsom, Surrey, and complied with the following specification: Specific gravity (at 20°C), 0·984–0·988; refractive index (n_D^{20}), 1·484–1·490; acidity (as phthalic acid), max 0·01%; water content, max 0·1%; ash, max 0·01%. This conforms to the specification of the British Standards Institution (1962).

Animals and diet. Rats of the CD strain (Sprague–Dawley-derived) were obtained from Charles River (UK) Ltd., Margate, Kent. They were housed in an animal room maintained at 20 ± 1°C with a relative humidity of 50–70% and given ground Spillers' Laboratory Small Animal Diet and tap water *ad lib.*

Experimental design and conduct

Short-term feeding study. Groups of fifteen male and fifteen female rats were given diets containing 0 (control), 0·2, 1·0 or 2·0% DEHP for 17 wk. Additional groups of five rats of each sex of similar body weight and from the same batch were given diets containing 0, 1·0 or 2·0% DEHP for 2 or 6 wk. The animals were observed frequently for abnormalities of condition or behaviour and were weighed initially, on days 1, 2, 6, 9, 13, 16 and 20 and then at weekly intervals up to day 120. The consumption of food and water was measured over the 24-hr period preceding each day of weighing.

Urine was collected during the last 2 days of treatment and examined for appearance, microscopic constituents and content of albumin, glucose, ketones, bile salts and blood. Renal concentrating and diluting ability was assessed at the same intervals by measuring the specific gravity and volume of the urine produced during a 6-hr period of water deprivation and in a 2-hr period after a water load of 25 ml/kg. In addition, at wk 6 and 17, the same measurements were made on the urine produced during a 4-hr period following 16 hr without water. A count of the number of cells in the urine was made using the 2-hr sample.

At the end of the appropriate period of feeding the animals were killed by exsanguination from the aorta under barbiturate anaesthesia following a 24-hr period without food. An autopsy was carried out during which any macroscopic abnormalities were noted and the brain, heart, liver, stomach, small intestine, caecum, spleen, kidneys, adrenal glands, gonads,

pituitary and thyroid were weighed. Samples of these organs and of lung, salivary gland, trachea, oesphagus, aorta, thymus, various lymph nodes, urinary bladder, colon, rectum, pancreas, uterus, spinal cord and skeletal muscle were preserved in 10% buffered formalin. Paraffin-wax sections of these tissues were stained with haematoxylin and eosin for microscopic examination.

Blood taken at autopsy was examined for haemoglobin concentration, packed cell volume and counts of erythrocytes and leucocytes. Slides were prepared from all blood samples for counting of reticulocytes and the different types of leucocytes. Serum from the blood samples taken at wk 17 was analysed for the content of urea, glucose, total protein, and for the activities of glutamic–pyruvic transaminase, glutamic–oxalacetic transaminase and lactic dehydrogenase.

Paired-feeding study. Two groups of ten male rats, caged individually, were given diets containing either 0 (control) or 2% DEHP for 120 days. The groups consisted of rats from the same litters and each treated rat was paired with a litter-mate control. The treated rats were fed *ad* lib. and each control rat was given a quantity of diet equal to that consumed during the previous day by its treated partner. The rats were weighed at intervals.

RESULTS

Rats receiving the highest dietary level (2·0%) of DEHP showed a loss of fur over the head and ventral surface of the body. This became evident during wk 1 of feeding and persisted throughout the study. By wk 17 these rats were emaciated and all of the females, but only two of the males, showed extensive loss of fur. One female rat from the group given the 2% diet was found dead on day 119. There was blood around the nose and mouth and the lungs appeared heavily congested, but autolysis precluded histopathological examination.

The mean body weight of rats on the two higher dietary levels was consistently less than that of controls (Table 1), the differences being statistically significant from day 2 onwards in both sexes given 2% DEHP and from day 6 in males given 1%, but only from day 83 in the females on 1%. At the end of the study, the body weights of the males and females fed 2% DEHP were, respectively, 71 and 61% of the control values. The weight of males, but not females, fed 0·2% DEHP was lower than that of the controls, but not to a statistically significant degree. All the DEHP-treated male rats and the females on the highest dietary level consumed less food than the controls, the difference being most pronounced over the first 48 hr of the study (Table 2). Over the whole experimental period the mean food intake was depressed to a statistically significant degree only in the rats given 2% DEHP. In the paired-feeding study the weight gain of the rats fed 2% DEHP was significantly less than that of their pair-fed controls despite the fact that the treated rats consumed slightly more food (Table 3). Water consumption (Table 1) was lower in male rats ingesting 1 or 2% DEHP and in females fed the 2% level than in the appropriate controls. The difference in mean intake over the whole experimental period was reduced to a statistically significant degree only in the male rats fed the 2% level. Mean water consumption was significantly greater than that of the controls in the female rats given 0·2 or 1% DEHP. The calculated intakes of DEHP are shown in Table 2.

The haemoglobin concentration, packed cell volume and erythrocyte count of the male rats given 1% DEHP were all approximately 10% less than the controls at wk 2 (Table 4). These differences were evident neither in the males receiving 2% DEHP nor in either group at wk 6. At wk 17 the haemoglobin concentration and packed cell volume were slightly but significantly lower than the control values in the male rats ingesting the 1 or 2% DEHP diets, although the erythrocyte count was unaffected. In the female rats the packed cell volume and haemoglobin concentration were slightly lower than the controls. These differences were statistically significant only for the packed cell volume at wk 6 and 17. At no time were there any statistically significant increases in the percentages of reticulocytes. Since there were no statistically significant differences between control and treated rats in the total leucocyte count, it was considered unnecessary to carry out the differential counts. The results of the serum analyses were similar in both treated and control rats.

There were no abnormal constituents in the urine. The cell excretion by the treated rats was not statistically greater than that in the control rats at any of the examinations (Table 5). At wk 2 the male rats given 1 or 2% DEHP produced urine that was slightly more concentrated, during a 6-hr period of water deprivation, than that of the controls. Similar results were obtained at wk 6, at which stage the volume of urine produced was also significantly lower at the highest dietary level. However, there were no statistically significant differences in either the concentration or volume of the 16–20-hr urine sample. Neither were there any statistically significant differences between control and treated rats in any of the renal function tests carried out in the males at wk 17 or in the females at wk 2. At both wk 6 and 17, the concentration of the 0–6 hr urine sample in the female rats receiving 2% DEHP was similar to the control value, but that of the 16–20-hr samples was significantly lower. The volume of the latter samples was significantly increased at wk 17. Additionally, at wk 17, this group excreted a smaller volume of more concentrated urine in the 2 hr following a water load.

Apart from the reduced size and loss of fur from some animals described earlier, no gross abnormalities were seen at autopsy of the female rats treated with DEHP. However, in the male rats fed diets containing 1 or 2% DEHP the testes were flaccid and visibly reduced in size. This effect was evident as early as wk 2 in rats given 2% DEHP, but not until wk 6 in the rats receiving 1% DEHP. At the lowest treatment level, 0·2%, testicular changes were not grossly apparent at any stage.

Except for the liver weights which were greater, no differences from the controls were seen in the organ weights of the rats receiving 0·2% DEHP. At the two higher dietary levels the weights of most organs, with the exception of the liver, were significantly reduced (Table 6). In particular, the weights of the testes were markedly decreased. Because of the reduced body

Table 1. *Mean body weights and water intake of rats fed diets containing 0–2·0% DEHP for up to 17 wk*

Dietary level (%)	Body weight (g) at day						Water intake (ml/rat/day) at day						Mean water intake (ml/rat/day)
	0†	1	27	55	90	120	0†	1	27	55	90	120	
Males													
0·0	96	105	340	478	569	628	18·3	18·5	37·1	38·0	28·5	26·3	30·1
0·2	98	105	325	455	539	588	17·6	19·7	37·3	36·3	32·3	24·7	30·1
1·0	98	100	297**	417***	493***	546***	18·0	15·1	34·3	27·9**	29·0	27·7	27·8
2·0	99	99	187***	300***	413***	447***	18·4	15·7	24·9**	30·9	34·4**	26·9	25·7*
Females													
0·0	85	92	214	273	309	329	15·9	15·7	21·5	21·9	18·1	19·4	22·3
0·2	88	95	216	277	308	325	17·9	18·9	24·9	34·5	26·5	22·1	25·0*
1·0	87	90	210	259	284*	297**	17·4	15·4	26·6*	24·5	25·9*	22·6	24·3*
2·0	88	87	131***	164***	191***	201***	18·3	14·8	21·1	21·3	18·9	16·7	19·7

†First day of treatment.

Body weights are the means for 15 animals. Values for water consumption are the means for three cages of five animals and were measured over the 24-hr period preceding the day shown. Figures marked with asterisks differ significantly from those of controls: $*P < 0{\cdot}05$; $**P < 0{\cdot}01$; $***P < 0{\cdot}001$. The methods used were Student's t test for body weight, ranking method of White (1952) for mean water intake and the Lord (1947) range test for weekly water intake.

Table 2. *Food intake and calculated intake of DEHP by rats fed diets containing 0–2·0% DEHP for up to 17 wk*

Dietary level (%)	Food intake (g/rat/day) at day						Mean food intake (g/rat/day)	DEHP intake‡ (mg/kg/day) at day					Mean DEHP intake (mg/kg/day)
	0†	1	27	55	90	120		1	27	55	90	120	
Males													
0·0	12·3	13·7	28·7	29·7	25·2	24·1	23·8	—	—	—	—	—	—
0·2	13·1*	14·0	26·2	24·9	23·4	21·2	21·4	268	161	109	87	72	143
1·0	13·7*	10·9**	26·7	24·5*	24·6	22·8	21·3	1090	899	588	499	418	737
2·0	13·9	8·7**	16·7**	23·1*	23·4**	22·1	17·3**	1754	1786	1540	1133	989	1440
Females													
0·0	11·5	10·1	18·3	15·9	15·6	15·4	15·6	—	—	—	—	—	—
0·2	11·9	10·9	16·6	17·6	16·1	16·2	16·2	229	154	127	105	100	154
1·0	11·6	8·9	18·2	16·2	18·1	17·3	16·1	984	867	625	637	582	797
2·0	11·9	5·8**	12·1**	11·6**	11·5**	7·5**	10·1**	1333	1847	1415	1204	746	1414

†First day of treatment.

‡Calculated from data on body weight and food intake.

Values for food intake are the means for three cages of five animals and were measured over the 24-hr period preceding the day shown. Those marked with asterisks differ significantly from controls. $*P < 0{\cdot}05$, $**P < 0{\cdot}01$. The methods used were the ranking method of White (1952) for the overall mean values and the Lord (1947) range test for the individual values.

Table 3. *Mean body weights of male rats fed diet containing 2% DEHP and of pair-fed controls*

Treatment	Body weight (g) at day 0†	20	51	83	106	120	Weight gain (g)	Total food consumed (g)
Pair-fed control	95	171	342	438	479	493	398	2268
2% DEHP	91	168	305	380*	419**	429**	338**	2346

†First day of feeding.
Values are means for groups of ten rats and those marked with asterisks differ significantly (ranking method of White, 1952) from those of the controls: $*P < 0.05$; $**P < 0.01$.

weight of the treated rats some of the differences in organ weight were eliminated when weights were expressed relative to body weight (Table 7). The most pronounced changes in relative organ weight were the increase in liver weight in rats on all dietary levels of DEHP and the decrease in testis weight in those on the two highest levels. There were consistent increases in the relative weights of the stomach, small intestine, caecum, kidney, heart and brain in rats of both sexes fed the higher dietary levels of DEHP, and many of these increases were statistically significant. In addition, the relative weights of the adrenal and thyroid glands tended to be increased at the 2% level and the relative pituitary weight was significantly increased at this level in the male rats at wk 17.

Histological examination of the tissues preserved at autopsy revealed some thickening of the alveolar walls in the lungs, but this finding was similar in incidence and severity in both treated and control animals. In the treated male rats there were histopathological changes in the testes, the incidence and severity of which were related to the dietary level of DEHP (Table 8). These changes were evident as early as wk 2. In severely affected testes the diameter of the seminiferous tubules was markedly reduced and the germinal epithelium consisted only of Sertoli cells, spermatogonia and occasionally a few primary spermatocytes. Less severely damaged testes contained up to about 50% of tubules showing these changes. In the remainder of the tubules in these testes the epithelium was of normal thickness with spermatids at various stages of development, but very few tubules contained mature sperm. This degree of damage corresponds to the category 'moderate' in Table 8. Where the degree of damage was slight, most tubules appeared normal, although the numbers of maturation-phase spermatids and mature sperm were reduced. Occasional tubules showed extensive loss of germinal epithelium. The interstitial cells of the testis

Table 4. *Mean haematological values for rats fed diets containing 0–2·0% DEHP for 2, 6 or 17 wk*

Sex and dietary level (%)	No. of rats examined	Hb (g/100 ml)	PCV (%)	RBC (10^6/mm^3)	Retics (% of RBC)	WBC (10^3/mm^3)
Male			**Wk 2**			
0	5	14·8	45	6·26	2·0	7·2
1·0	5	13·0**	40	5·77**	2·7	5·6
2·0	5	14·8	42	6·48	1·3	5·3
Female						
0	5	15·1	43	6·50	1·6	4·9
1·0	5	14·4	38	6·24	2·2	4·2
2·0	5	14·8	41	6·82	0·8*	6·4
Male			**Wk 6**			
0	5	15·1	48	7·08	1·4	6·2
1·0	5	15·0	45	6·96	1·6	6·0
2·0	5	14·9	46	6·86	1·3	5·0
Female						
0	5	15·8	49	7·81	1·2	6·9
1·0	5	15·4	44***	7·90	1·0	4·7
2·0	5	14·4	43***	7·49	1·2	4·5
Male			**Wk 17**			
0	15	16·0	46	7·57	0·9	6·4
0·2	15	15·4	45	7·44	0·6	7·5
1·0	15	14·5*	43**	6·97	0·8	6·5
2·0	15	14·5*	43***	7·60	0·9	6·5
Female						
0	15	14·9	45	7·14	0·9	4·7
0·2	15	14·9	44	7·05	0·8	4·4
1·0	15	14·4	42*	7·26	1·0	5·4
2·0	15	13·8	42**	6·78	0·8	5·5

Hb = Haemoglobin PCV = Packed cell volume RBC = Red blood cells
Retics = Reticulocytes WBC = White blood cells
The figures are means for the numbers of rats shown and those marked with asterisks differ significantly (Student's *t* test) from those of the controls: $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

Table 5. *Results of renal concentration tests and urinary cell counts for rats fed diets containing 0–2·0% DEHP for 2, 6 or 17 wk*

Sex and dietary level (%)	No. of rats examined	Cells (10^3/hr)	Concentration test: Specific gravity 0–6 hr	Specific gravity 16–20 hr	Volume (ml) 0–6 hr	Volume (ml) 16–20 hr	Dilution test (2 hr): Specific gravity	Volume (ml)
Male				Wk 2				
0	5	1·6	1·013	—	2·7	—	1·024	0·8
1·0	5	0·6	1·033	—	1·8	—	1·023	1·7
2·0	5	0·3	1·022*	—	2·1	—	1·023	0·6
Female								
0	5	0·5	1·030	—	1·4	—	1·028	0·4
1·0	5	0·4	1·028	—	1·8	—	1·017	1·4
2·0	5	0·4	1·023	—	2·1	—	1·018	1·0
Male				Wk 6				
0	5	0·6	1·017	1·075	3·7	0·3	1·017	3·3
1·0	5	0·6	1·038**	—	3·2	—	1·015	4·8
2·0	5	0·1*	1·045**	1·066	1·5**	0·5	1·025	2·0
Female								
0	5	1·4	1·021	1·072	2·3	0·5	1·011	3·8
1·0	5	0·6	1·033	1·079	1·8	0·4	1·008	3·9
2·0	5	0·1**	1·019	1·034*	2·0	0·6	1·007	2·6
Male				Wk 17				
0	12	1·6	1·025	1·068	4·2	0·5	1·005	8·0
0·2	12	2·1	1·025	1·068	3·9	1·1	1·006	9·7
1·0	12	1·7	1·027	1·076	3·2	0·6	1·008	8·2
2·0	12	1·9	1·031	1·078	4·1	0·9	1·007	7·2
Female								
0	12	0·7	1·026	1·076	2·5	0·3	1·005	5·6
0·2	12	1·1	1·015	1·072	2·2	0·3	1·005	5·5
1·0	12	0·4	1·044	1·073	2·3	0·6	1·007	4·9
2·0	12	0·5	1·034	1·037**	1·4	1·3*	1·012**	3·8*

The figures are means for the numbers of rats shown and those marked with asterisks differ significantly (ranking method of White, 1952) from those of the controls: $*P < 0·05$; $**P < 0·01$.
A dash indicates that the measurement was not carried out.

appeared histologically normal in all affected rats. In addition to the testicular lesion, there was a dose-related incidence of pituitary changes in the male rats killed at wk 17. The basophils of the pars distalis were enlarged and contained a large, clear central vacuole. There were no histopathological changes in the female gonads or pituitary glands.

DISCUSSION

The reduced rate of body-weight gain in both sexes of rat ingesting 2% DEHP and in the male rats given 1% DEHP was associated with a decrease in food consumption. This depression of food consumption was most pronounced during the very early stages of the study, a pattern of food intake often associated with a diet that is unpalatable to rats. The paired-feeding study showed this to be partly true, since the difference in weight between rats given 2% DEHP and pair-fed controls was only 60 g compared with a 180-g difference between these groups in the *ad lib.* fed animals. However, the failure of the DEHP-treated rats to gain weight at the same rate as the pair-fed controls showed that unpalatability of the diet did not account entirely for the reduced rate of body-weight gain.

At wk 17 there were dose-related decreases in the haemoglobin concentrations and packed cell volumes in both sexes. Similar effects were observed in the female rats at wk 6 and in the males fed a diet containing 1% DEHP at wk 2. The apparent relation to dose suggests that the effects observed were due to the DEHP treatment. However, the present experiment does not allow any conclusions to be drawn concerning the aetiology of these changes. There was no evidence of splenic haemosiderosis, suggesting that they were not related to extravascular erythrophagocytosis of damaged cells. Since the proportion of reticulocytes was unaltered there was no evidence of any compensated haemorrhagic or haemolytic process nor of any direct effect on erythropoiesis. At present these findings remain unexplained, but they deserve further investigation.

A number of the changes in absolute and relative organ weight were more probably due to the markedly reduced body weights of the treated rats than to specific effects of DEHP. Similar decreases in absolute and increases in relative weights of the brain, stomach, small intestine, adrenals and thyroid have been noted in rats fed restricted quantities of control diet (Feron, de Groot, Spanjers & Til, 1973; Peters & Boyd, 1966; Schwartz, Tornaben & Boxhill, 1973) and in toxicity studies with other materials that have resulted in reduced body-weight gain (Carpanini, Gaunt, Hardy, Gangolli, Butterworth & Lloyd, 1978; Gaunt, Sharratt, Grasso, Lansdown & Gangolli, 1974). The reduction in spleen weight was no longer evident when the weight was expressed relative to body weight and was probably also attributable to the lower body weight of the treated rats. Similar

Table 6. *Organ weights of rats fed diets containing 0–2·0% DEHP for 2, 6 or 17 wk*

Sex and dietary level (%)	No. of rats examined	Organ weight (g)												Body weight (g)
		Brain	Heart	Liver	Spleen	Kidneys	Stomach	Small intestine	Caecum	Adrenals†	Gonads‡	Pituitary†	Thyroid†	
Male							**Wk 2**							
0	5	1·95	0·81	5·54	0·52	1·65	1·23	5·97	0·95	35·0	1·90	7·2	11·7	176
1·0	5	1·90	0·69	10·16***	0·53	1·66	1·13	6·09	0·90	32·2	1·48	6·4	13·6	164
2·0	5	1·81	0·44***	6·83	0·26**	1·11***	1·04*	4·62	0·71	28·4	0·62***	4·4*	12·3	106**
Female														
0	5	1·80	0·68	5·42	0·44	1·61	1·04	5·44	0·84	54·0	86	8·1	12·3	151
1·0	5	1·77	0·61	7·76***	0·40	1·46	0·95	5·55	0·83	38·0**	73	7·1	13·7	147
2·0	5	1·71	0·34***	5·67	0·20***	0·90***	0·83*	3·68***	0·55***	28·2***	33**	3·5***	9·2	86***
Male							**Wk 6**							
0	5	2·10	1·32	11·24	0·81	2·94	1·69	9·06	1·55	46·2	3·25	9·2	13·7	376
1·0	5	1·98	1·29	15·12**	0·62	2·53*	1·50	10·06	1·52	35·6	0·95***	8·6	15·0	320**
2·0	5	1·88	0·81**	12·57	0·52*	1·62***	1·42*	8·14	1·06*	35·7	0·81***	6·3	14·6	189***
Female														
0	5	1·93	0·86	6·92	0·63	1·91	1·40	6·79	1·04	54·2	133	10·7	15·0	241
1·0	5	1·90	0·80	9·46**	0·52	1·76	1·29	6·78	1·10	50·0	132	11·6	15·0	215
2·0	5	1·70**	0·44***	6·19	0·27***	1·01***	1·04**	4·03***	0·55**	29·2***	41***	4·9**	10·6*	106***
Male							**Wk 17**							
0	15	2·26	1·65	14·16	0·87	3·40	1·84	8·84	1·54	53·9	3·65	11·5	23·1	615
0·2	15	2·22	1·54	15·81*	0·86	3·39	1·81	8·93	1·38	54·4	3·49	11·4	21·8	579
1·0	15	2·24	1·49*	18·38***	0·77*	3·38	1·78	8·80	1·31*	53·4	2·17***	12·2	26·4	532**
2·0	15	2·13*	1·31***	18·10***	0·71***	3·04**	1·67*	8·31	1·27*	46·1	1·00***	12·3	22·0	440***
Female														
0	15	2·09	0·98	7·35	0·52	1·85	1·27	6·54	0·96	55·9	99	13·4	24·2	327
0·2	15	2·07	1·00	8·55*	0·54	1·97	1·27	6·82	1·05	61·1	95	12·9	21·1	329
1·0	15	2·06	0·93	10·32***	0·52	1·95	1·25	7·17	1·01	65·3	108	13·8	23·1	301
2·0	15	1·96***	0·75***	8·67*	0·35***	1·33***	1·28	5·82	0·83	37·5***	49***	7·6***	16·6*	193***

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

Table 7. *Relative organ weights of rats fed diets containing 0–2·0% DEHP for 2, 6 or 17 wk*

Sex and dietary level (%)	No. of rats examined	Relative organ weight (g/100 g body weight)											
		Brain	Heart	Liver	Spleen	Kidneys	Stomach	Small Intestine	Caecum	Adrenals†	Gonads‡	Pituitary†	Thyroid†
						Wk 2							
Male													
0	5	1·12	0·46	3·15	0·30	0·95	0·70	3·39	0·54	20·1	1·08	4·1	6·6
1·0	5	1·17	0·42**	6·19***	0·32	1·01	0·70	3·76	0·55	19·9	0·90	3·9	8·3
2·0	5	1·75	0·42*	6·47***	0·24*	1·05	1·01**	4·41**	0·67**	26·9*	0·59**	4·0	12·0**
Female													
0	5	1·19	0·45	3·61	0·29	1·07	0·69	3·62	0·56	36·0	57	5·4	8·2
1·0	5	1·21	0·42*	5·28***	0·27	1·00	0·65	3·79	0·57	25·8**	50	4·8	9·3
2·0	5	2·04***	0·40*	6·62***	0·24*	1·06	0·98**	4·39	0·65	33·1	41	4·1	10·1
						Wk 6							
Male													
0	5	0·56	0·35	2·99	0·21	0·78	0·45	2·42	0·42	12·3	0·88	2·6	3·6
1·0	5	0·62	0·40*	4·72***	0·19	0·79	0·47	3·15*	0·47	11·1	0·30***	2·4	4·7
2·0	5	1·03**	0·43***	6·76***	0·28	0·86	0·78**	4·58*	0·54	20·0*	0·44***	3·0	8·1
Female													
0	5	0·80	0·36	2·88	0·26	0·80	0·58	2·80	0·43	22·5	55	4·5	6·2
1·0	5	0·89	0·38	4·40***	0·24	0·82	0·60	3·16	0·51	23·0	61	5·4	7·1
2·0	5	1·63	0·41*	5·83***	0·25	0·95**	0·98***	3·80***	0·52	27·7	39*	4·7	10·1**
						Wk 17							
Male													
0	15	0·37	0·27	2·31	0·14	0·56	0·30	1·45	0·25	8·9	0·60	1·9	3·8
0·2	15	0·39	0·27	2·72***	0·15	0·59	0·31	1·55	0·24	9·5	0·61	1·9	3·4
1·0	15	0·42**	0·28	3·45***	0·14	0·64**	0·33**	1·66*	0·25	10·1	0·41***	2·3	5·1
2·0	15	0·50***	0·30*	4·12***	0·16	0·70***	0·38***	1·91***	0·29*	10·9	0·23***	2·8***	5·1
Female													
0	15	0·64	0·30	2·25	0·16	0·57	0·39	2·01	0·29	17·2	30	4·2	7·5
0·2	15	0·64	0·30	2·60*	0·17	0·60	0·39	2·09	0·32	19·0	29	4·0	6·5
1·2	15	0·69	0·31	3·46**	0·17	0·65***	0·42	2·40	0·34*	21·9**	37	4·6	7·8
2·0	15	1·10***	0·39***	4·59***	0·19*	0·71***	0·72***	3·13***	0·44***	20·4	25	4·0	9·1

†Relative weights of these organs 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 asterisks differ significantly (Student's t test) from those of the controls: *$P < 0·05$; **$P < 0·01$; ***$P < 0·001$.

Table 8. *Incidence and severity of testicular damage and incidence of 'castration cells' in the pituitary glands of rats fed diets containing 0–2·0% DEHP for 2, 6 or 17 wk*

Dietary level (%)	No. of rats examined	No. of rats affected				
		Severity of testicular damage†			Total incidence of testicular damage	'Castration cells' in pituitary
		Slight	Moderate	Severe		
Wk 2						
0	5	0	0	0	0	0
1·0	5	3	1	0	4	0
2·0	5	0	0	5	5*	0
Wk 6						
0	5	0	0	0	0	0
1·0	5	1	1	2	4	0
2·0	5	0	0	5	5*	0
Wk 17						
0	15	0	0	0	0	0
0·2	15	4	0	0	4	1
1·0	15	5	5	2	12***	4
2·0	15	0	5	10	15***	9

†For description of lesions see text.

Figures marked with asterisks among the total incidence of testicular damage differ significantly by the chi-square test from the controls: $^{*}P < 0{\cdot}05$; $^{***}P < 0{\cdot}001$.

changes in spleen weight in partially starved rats were reported by Feron *et al.* (1973) and Schwartz *et al.* (1973).

The relative kidney weight in the treated rats showed a dose-related increase at both wk 6 and 17, a finding in keeping with the results of previous studies with DEHP (Carpenter *et al.* 1953; Harris *et al.* 1956; Nikonorow *et al.* 1973). Although there was no histological evidence of damage in the enlarged kidneys, there were some differences between treated and control rats in the results of the renal function tests. Male rats fed 2% DEHP for 6 wk excreted reduced volumes of more concentrated urine in the concentration and dilution test. However, since this effect was not evident at wk 17 it is more probable that it related to the reduced water intake of these rats during the early part of the study than to a specific effect of DEHP. However, there was an indication of a reduced renal concentrating and diluting ability in the female rats given 2% DEHP for 17 wk suggesting some degree of functional impairment. On this basis it would be reasonable to postulate that prolonged exposure to high dose levels of DEHP may have adverse effects on the kidney.

An increase in the relative weight of the empty caecum has been reported in rats fed restricted amounts of food (Peters & Boyd, 1966). The increase in relative caecum weight in the DEHP-treated rats may have a similar basis, but the possibility that it reflects a specific effect of DEHP is raised by the observation that caecal enlargement is also caused by di-*n*-butyl phthalate under conditions that do not lead to a significant reduction in body weight (T. J. B. Gray, unpublished results 1975). The toxicological significance of such caecal enlargement remains a matter of debate (Butterworth, Lake, Mason & Rowland, 1975).

It is likely that the increase in relative heart weight in the DEHP-treated rats is also attributable to the effects of the reduced body weight, since there were no histological changes in the heart, and increases in relative heart weight in rats ingesting restricted amounts of food were reported by Carpanini *et al.* (1978) and Peters & Boyd (1966), although Feron *et al.* (1973), Gaunt *et al.* (1974) and Schwartz *et al.* (1973) found no such effect under similar conditions. On the other hand, the possibility of a specific effect on heart tissue cannot be excluded in view of the findings of Stein, Caasi & Nair (1974) that the concentration of DEHP in the heart of rats fed 0·1% DEHP was some four times that in the liver, and of those of Hillman *et al.* (1975) that heart tissue from infants with PVC umbilical catheters contained significant quantities of DEHP.

The increases in relative liver weight in the treated rats at all dietary levels from wk 2 onwards were not associated with any histological evidence of liver damage. The production of an enlarged but histologically normal liver has been reported in previous studies with DEHP (Carpenter *et al.* 1953; Harris *et al.* 1956). This type of liver effect is produced by a wide variety of substances and is frequently associated with an increase in the activity of the hepatic microsomal drug-metabolizing enzymes (Conney, 1967; Schulte-Hermann, 1974). In such circumstances it has been regarded as an adaptive, physiological response of the liver to increased metabolic demand (Golberg, 1966). However, DEHP does not cause induction, but rather an inhibition of drug-metabolizing enzyme activity (Lake, Gangolli, Wright, Grasso & Lloyd, 1974). Recent studies have provided evidence that this situation, namely liver enlargement unaccompanied by induction of drug-metabolizing enzymes, is indicative of incipient hepatotoxicity (Crampton, Gray, Grasso & Parke, 1977; Grasso, Wright, Gangolli & Hendy, 1974; Gray, 1975). Additional evidence that the liver enlargement produced by DEHP reflects an adverse effect of the compound derives from the studies of Lake *et al.* (1975) who reported mitochondrial swelling and dilatation of both smooth and rough endoplasmic reticulum in the livers of rats given large oral doses of DEHP.

The weight of the gonads was reduced in both sexes

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of rats receiving DEHP. In the male rats this decrease was equally evident on a relative-weight basis and was accompanied by histological evidence of dose-related testicular damage. At the highest dose level, most seminiferous tubules showed extensive loss of germinal epithelium. In addition, cells fitting the description of 'castration cells' (Russfield, 1967) were present in the pituitaries of male rats treated with DEHP for 17 wk. The incidence of rats with these cells was dose-related and the relative weight of the pituitary was significantly increased at the highest dose level. The presence of 'castration cells', which typically appear following gonadectomy, is considered to be a sensitive indicator of gonadal deficiency (Russfield, 1967). These findings indicate that, under the conditions of the present study, DEHP produces testicular damage in the rat. Similar findings at comparable dietary levels of DEHP have been reported previously by Shaffer *et al.* (1945). In the present study, the degree of testicular damage produced at the lowest dose level of DEHP was slight and the incidence was not statistically significant. Testicular changes similar to those produced by DEHP have been observed with an incidence of 5–8% in untreated rats of the same age and strain as those used in the present study (BIBRA unpublished data, 1974). Thus it cannot be stated unequivocally that the testicular damage in the rats receiving 0·2% DEHP was related to treatment. However, the overall dose relationship of the testicular damage in the treated rats, the absence of any testicular changes in the control rats in this experiment and the presence of 'castration cells' in the pituitary of one rat given 0·2% DEHP suggests that the testicular damage at this level was related to treatment.

In the female rats the decreases in ovary weight were much less evident when the organ weights were expressed relative to body weight. Moreover, there were no histological abnormalities in the ovary or the pituitary and the relative pituitary weight was unaffected. These considerations suggest that DEHP may produce gonadal damage only in the male rat and that the decreases in ovary weight were primarily a reflection of the reduced body weight of the treated rats.

At present it is not known whether DEHP (or, more probably, its metabolites) exerts its effects directly on the testis or whether it acts indirectly, possibly by interfering with the hormonal control of normal testicular development and function. It is noteworthy, however, that in the present study, histological changes in the pituitary occurred only at a relatively late stage. In view of this observation and the apparent lack of effect of DEHP on the female gonads, it is improbable that DEHP acts primarily at the level of the pituitary. It is also unlikely that the reduction in food intake evoked by DEHP is relevant to the production of the testicular damage. Studies in which rats have been subjected to similar or greater degrees of dietary restriction have failed to produce any evidence of testicular damage (Gaunt, Hardy, Grasso, Gangolli & Butterworth, 1976). Investigations to elucidate the mechanisms by which DEHP produces testicular damage are currently in progress in the BIBRA laboratories.

Earlier views on the safety-in-use of DEHP as a plasticizer in packaging for direct food contact have been based on the collected findings of studies reported by Carpenter *et al.* (1953) and Harris *et al.* (1956). These established that at 0·1% in the diet of rats no untoward effects attributable to the feeding of DEHP were apparent after 2 yr. This corresponds to an acceptable dietary intake of the order of 30 mg/day for a 60-kg adult, after application of the traditional 100-fold safety factor. The present investigations demonstrate that in rats fed 0·2% DEHP in the diet for periods up to 17 wk, effects on the liver and testes were discernible. It must be considered unfortunate that no definitive information is available on the extent to which this plasticizer migrates from packaging into food under various storage conditions. Consequently, the significance of these findings in relation to human exposure remains to be determined. In the interim, continuing reliance must be placed on the work of Harris *et al.* (1956) as the basis for assessing safe human intakes.

REFERENCES

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

Albro, P. W., Thomas, R. O. & Fishbein, L. (1973). Metabolism of diethylhexyl-phthalate by rats. Isolation and characterisation of urinary metabolites. *J. Chromat.* **76**, 321.

Autian, J. (1973). Toxicity and health threats of phthalate esters: Review of the literature. *Envir. Hlth. Perspect.* no. 4, p. 3.

British Standards Institution (1962). Specification for Di-(2-ethylhexyl) Phthalate. BS 1995:1962.

Butterworth, K. R., Lake, B. G., Mason, P. L. & Rowland, I. R. (1975). Enlargement of the caecum in the rat. *Br. J. Pharmac.* **55**, 260P.

Calley, D., Autian, J. & Guess, W. L. (1966). Toxicology of a series of phthalate esters in rats. *J. Pharm. Sci.* **55**, 158.

Carpanini, F. M. B., Gaunt, I. F., Hardy, J., Gangolli, S. D., Butterworth, K. R. & Lloyd, A. G. (1978). Short-term toxicity of allyl alcohol in rats. *Toxicology*. In press.

Carpenter, C. P., Weil, C. S. & Smyth, H. F. (1953). Chronic oral toxicity of di-(2-ethylhexyl) phthalate for rats, guinea-pigs and dogs. *Archs ind. Hyg.* **8**, 219.

Conney, A. H. (1967). Pharmacological implications of microsomal enzyme induction. *Pharmac. Rev.* **19**, 317.

Crampton, R. F., Gray, T. J. B., Grasso, P. & Parke, D. V. (1977). Long-term studies on liver enlargement in the rat. II. Transient induction of microsomal enzymes leading to liver damage and nodular hyperplasia by safrole and Ponceau MX. *Toxicology*. 7. 307.

Daniel, J. W. & Bratt, H. (1974). The absorption, metabolism and tissue distribution of di-(2-ethylhexyl) phthalate in rats. *Toxicology* **2**, 51.

Fassett, D. W. (1967). Esters. In *Industrial Hygiene and Toxicology. Vol. II. Toxicology.* Edited by F. A. Patty. 2nd Ed. p. 1908. Interscience, New York.

Feron, V. J., de Groot, A. P., Spanjers, M. T. & Til, H. P. (1973). An evaluation of the criterion "organ weight" under conditions of growth retardation. *Fd Cosmet. Toxicol.* **11**, 85.

Fishbein, L. & Albro, P. W. (1972). Chromatographic and biologic aspects of the phthalate esters. *J. Chromat.* **70**, 365.

Gaunt, I. F., Hardy, J., Grasso, P., Gangoli, S. D. & Butterworth, K. R. (1976). Long-term toxicity of cyclohexylamine hydrochloride in the rat. *Fd Cosmet. Toxicol.* **14**, 255.

Gaunt, I. F., Sharratt, M., Grasso, P., Lansdown, A. B. G. & Gangolli, S. D. (1974). Short-term toxicity of cyclohexylamine hydrochloride in the rat. *Fd Cosmet. Toxicol.* **12**, 609.

Golberg, L. (1966). Liver enlargement produced by drugs: Its significance. *Proc. Eur. Soc. Study Drug Toxicity.* **7**, 171.

Grasso, P., Wright, M. G., Gangolli, S. D. & Hendy, R. J. (1974). Liver response tests. IX. Cytopathological changes in the enlarged but histologically normal rat liver. *Fd Cosmet. Toxicol.* **12**, 341.

Gray, T. J. B. (1975). Studies on Chemically-induced Liver Enlargement and Hepatic Nodular Lesions in the Rat. Ph.D. Thesis. University of Surrey.

Harris, R. S., Hodge, H. C., Maynard, E. A. & Blanchet, H. J. (1956). Chronic oral toxicity of 2-ethylhexyl phthalate in rats and dogs. *A.M.A. Archs ind. Hlth* **13**, 259.

Hillman, L. S., Goodwin, S. L. & Sherman, W. R. (1975). Identification and measurement of plasticizer in neonatal tissues after umbilical catheters and blood products. *New Engl. J. Med.* **292**, 381.

Hodge, H. C. (1943). Acute toxicity for rats and mice of 2-ethylhexanol and 2-ethylhexyl phthalate. *Proc. Soc. exp. Biol. Med.* **53**, 20.

Jaeger, R. J. & Rubin, R. J. (1970a). Contamination of blood stored in plastic packs. *Lancet* **ii**, 151.

Jaeger, R. J. & Rubin, R. J. (1970b). Plasticizers from plastic devices: Extraction, metabolism and accumulation by biological systems. *Science, N.Y.* **170**, 460.

Jaeger, R. J. & Rubin, R. J. (1972). Migration of a phthalate ester plasticizer from polyvinyl chloride blood bags into stored human blood and its location in human tissues. *New Engl. J. Med.* **287**, 1114.

Jones, A. E., Kahn, R. H., Groves, J. T. & Napier, E. A. (1975). Phthalate ester toxicity in human cell cultures. *Toxic. appl. Pharmac.* **31**, 283.

Lake, B. G., Gangolli, S. D., Grasso, P. & Lloyd, A. G. (1975). Studies on the hepatic effects of orally administered di-(2-ethylhexyl) phthalate in the rat. *Toxic appl. Pharmac.* **32**, 355.

Lake, B. G., Gangolli, S. D., Wright, M. G., Grasso, P. & Lloyd, A. G. (1974). Studies on the effects of the oral administration of di-(2-ethylhexyl) phthalate on some hepatic enzymes in the rat. *Biochem. Soc. Trans.* **2**, 322.

Lawrence, W. H., Malik, M., Turner, J. E., Singh, A. R. & Autian, J. (1975). A toxicological investigation of some acute, short-term and chronic effects of administering di-(2-ethylhexyl) phthalate (DEHP) and other phthalate esters. *Envir. Res.* **9**, 1.

Lord, E. (1947). The use of range in place of standard deviation in the *t*-test. *Biometrika* **34**, 41.

Marcel, Y. L. & Noel, S. P. (1970). Contamination of blood stored in plastic packs. *Lancet*, **i**, 35.

Mayer, F. L., Stalling, D. L. & Johnson, J. L. (1972). Phthalate esters as environmental contaminants. *Nature, Lond.* **238**, 411.

Morris, R. J. (1970). Phthalic acid in the deep sea jellyfish Atolla. *Nature, Lond.* **227**, 1264.

Nazir, D. J., Alcaraz, A. P., Bierl, B. A., Beroza, M. & Nair, P. P. (1971). Isolation, identification and specific localisation of di-(2-ethylhexyl) phthalate in bovine heart muscle mitochondria. *Biochemistry, N.Y.* **10**, 4228.

Neergaard, J., Nielsen, B., Faurby, V., Christensen, D. H. & Nielsen, O. F. (1971). Plasticizers in PVC and the occurrence of hepatitis in a haemodialysis unit. *Scand. J. Urol. Nephrol.* **5**, 141.

Nikonorow, M., Mazur, H. & Piekacz, H. (1973). Effect of orally administered plasticizers and polyvinylchloride stabilizers in the rat. *Toxic. appl. Pharmac.* **26**, 253.

Peters, J. M. & Boyd, E. M. (1966). Organ weights and water levels of the rat following reduced food intake. *J. Nutr.* **90**, 354.

Rowland, I. R. (1974). Metabolism of di-(2-ethylhexyl) phthalate by the contents of the alimentary tract of the rat. *Fd Cosmet. Toxicol.* **12**, 293.

Russfield, A. B. (1967). Pathology of the endocrine glands, ovary and testis of rats and mice. In *Pathology of Laboratory Rats and Mice*. Edited by E. Cotchin and F. J. C. Roe, p. 391. Blackwell Scientific Publications, Oxford.

Schulte-Hermann, R. (1974). Induction of liver growth by xenobiotic compounds and other stimuli. *Crit. Rev. Toxicol.* **3**, 97.

Schwartz, E., Tornaben, J. A. & Boxhill, G. A. (1973). The effects of food restriction on haematology, clinical chemistry and pathology in the albino rat. *Toxic. appl. Pharmac.* **25**, 515.

Shaffer, C. B., Carpenter, C. P. & Smyth, H. F. (1945). Acute and subacute toxicity of di-(2-ethylhexyl) phthalate with note upon its metabolism. *J. ind. Hyg. Toxicol.* **27**, 130.

Singh, A. R., Lawrence, W. H. & Autian, J. (1972). Teratogenicity of phthalate esters in rats. *J. pharm. Sci.* **61**, 51.

Singh, A. R., Lawrence, W. H. & Autian, J. (1974). Mutagenic and antifertility sensitivities of mice to di-(2-ethylhexyl) phthalate (DEHP) and dimethoxyethyl phthalate. *Toxic. appl. Pharmac.* **29**, 35.

Stalling, D. L., Hogan, J. W. & Johnson, J. L. (1973). Phthalate ester residues—their metabolism and analysis in fish. *Envir. Hlth Perspect.* no. 4, p. 53.

Stein, M. S., Caasi, P. I. & Nair, P. P. (1974). Influence of dietary fat and di-(2-ethylhexyl) phthalate on tissue lipids in rats. *J. Nutr.* **104**, 187.

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

Fd Cosmet. Toxicol. Vol. 15, pp. 401–410. Pergamon Press 1977. Printed in Great Britain

MORPHOLOGICAL CHANGES IN MONKEYS CONSUMING A DIET CONTAINING LOW LEVELS OF 2,3,7,8-TETRACHLORODIBENZO-*p*-DIOXIN

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Abstract—Female rhesus monkeys given a diet containing 500 ppt ($t = 10^{12}$) 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) for 9 months became anaemic within 6 months and pancytopenic after 9 months of exposure. The marked thrombocytopenia was associated with widespread haemorrhage. Death occurred in five of the eight animals between months 7 and 12 of the experiment at total exposure levels of 2–3 μg TCDD/kg body weight. At autopsy, in addition to the extensive haemorrhage, there was a distinct hypocellularity of the bone marrow and lymph nodes. Hypertrophy, hyperplasia and metaplasia of the epithelium in the bronchial tree, bile ducts, pancreatic ducts, salivary-gland ducts and palpebral conjunctivae were observed. Squamous metaplasia and keratinization of the sebaceous glands and hair follicles were present in the skin. Death was attributed to complications from the severe pancytopenia.

INTRODUCTION

2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) is produced in small amounts during the production of trichlorophenols. Uncontrolled chemical reactions in the presence of heat have led to the development of considerable amounts of TCDD in the commercial preparations. Explosions resulting from overheated chemical reactions have been responsible for human exposure to TCDD in Europe and the United States. One of the serious complications of this exposure has been persistent chloracne.

The health implications of TCDD, other than those related to industrial exposure, were brought to the attention of the general public during the Vietnam conflict. Defoliants widely used in the combat zones contained varying concentrations of TCDD, which has been held responsible for physical abnormalities that have developed in the human population (Rose & Rose, 1972). In addition to chloracne, exposed individuals have experienced fatigue, peripheral numbness (Rose & Rose, 1972), increased abortions and infant deaths, and liver cancer (Tung, 1973). More recently, serious physical problems, which included severe weight loss, acne, alopecia, haematurea and abortions, have appeared in horses that inhabited arenas sprayed with residues of trichlorophenol production containing TCDD (Carter, Kimbrough, Liddle, Cline, Zack & Barthel, 1975). The detection of minute amounts of dioxins in human milk (M. Meselson, personal communication 1977) and beef fat (R. T. Ross, personal communication 1976) have caused concern about the exposure of man and animals to TCDD. In the study now reported, emphasis is placed on determining the pathological alterations that occur in primates after low-level, long-term exposure to TCDD in food.

EXPERIMENTAL

Eight young healthy female rhesus monkeys weighing approximately 5·6 kg were placed on a diet of pelleted monkey food to which 500 ppt‡ TCDD (Dow Chemical Company, Midland, Mich.) had been added. The experimental diet was prepared in 100-lb lots by suspending TCDD (22·7 μg) in approximately 1 ml of acetone and adding this to 250 ml of corn oil; the corn oil was pre-mixed in 25 lb of finely ground monkey food (Purina Monkey Chow, Ralston-Purina Co., St. Louis, Mo.), and this mixture was then added to an additional 75 lb of food and mixed thoroughly for 10 min. The food was then pelleted and stored at −5°C until used. The monkeys remained on the experimental diet for 9 months. General health was monitored by daily observation, and in addition complete blood counts were made and serum protein, serum lipid, serum glutamic–pyruvic transaminase (SGPT) and blood-urea nitrogen were determined on a monthly basis. The food intake for each animal was monitored daily throughout the experiment.

A complete autopsy was performed on the monkeys that died. Sections of the tissues were fixed in 10% neutral buffered formalin, dehydrated, embedded in paraffin and sectioned at 5 μm. The sections were stained with haematoxylin and eosin and examined using a light microscope.

RESULTS

Clinical observations

Following 3 months of TCDD feeding (5·8 ± 0·9 μg total intake per animal) the monkeys developed periorbital oedema, loss of facial hair and eyelashes,

‡$t = 10^{12}$ throughout this paper.

Table 1. *Haemograms of monkeys fed 500 ppt TCDD for 9 months**

Animal no.	White blood cells ($\times 10^3$/mm^3)		Blood platelets ($\times 10^3$/mm^3)		Haemoglobin (gm%)		Haematocrit (%)	
	Initial	Terminal	Initial	Terminal	Initial	Terminal	Initial	Terminal
7†	11·0	2·3	240	23	13·5	4·0	41·0	12·0
9†	7·9	2·4	360	44	14·1	6·9	43·0	21·5
23	10·3	3·8	380	28	14·6	12·6	45·5	40·0
31	10·6	10·4	400	480	14·1	10·7	45·0	35·5
32‡	8·5	4·1	300	50	14·1	6·0	43·0	19·5
38†	9·5	3·8	330	34	13·6	6·6	43·0	22·0
41	7·8	8·0	350	340	13·1	11·5	40·0	44·5
49§	8·6	8·7	260	54	13·7	8·5	43·0	29·5

*Values obtained 3 months after the end of treatment unless otherwise stated.
†Died during wk 44 of experiment.
‡Died during wk 28 of experiment.
§Died during wk 36 of experiment.

accentuated hair follicles and dry scaly skin. These changes became more prominent in six of the eight animals by month 6 of exposure ($11·3 \pm 1·7$ μg total intake per animal). The majority of the animals showed a decrease in haemoglobin and haematocrit by month 6. The haematological changes became more accentuated in six of the seven animals that survived 9 months of TCDD exposure (Table 1). However, blood urea nitrogen, total serum lipid, serum cholesterol, SGPT, total serum protein and albumin/globulin ratios were not altered appreciably during the experiment except in terminal animals where there was a slight decrease in serum albumin and an increase in SGPT. The monkeys also lost weight throughout the experiment although their food intake was unaltered (Table 2).

Animal no. 32 became severely anaemic, thrombocytopenic and leucopenic in month 7 of exposure after a total intake of 11·2 μg (Table 1). Prior to death the peripheral blood smears were practically devoid of immature red and white blood cells. During the subsequent 4 months four additional animals (nos 7, 9, 38 and 49) showed similar clinical changes and died. Their peripheral leucocytes and platelets decreased to levels as low as 2300 and 24,000/mm^3 from normal values of 7–10,000 and 200–400,000/mm^3, respectively. The total TCDD intake of these animals at the time of death was $18·6 \pm 1·8$ μg/animal. Although all animals were removed from the TCDD diet after 9 months, the three animals (nos 23, 31 and 41) that survived for 12 months experienced a continuing loss of hair and periorbital oedema. One of the surviving monkeys (no. 23) developed a severe leucopenia and thrombocytopenia by month 12 (Table 1).

Table 2. *Body weights of monkeys fed 500 ppt TCDD for 9 months*

Animal no.	Body weight (kg)	
	Initial	Terminal*
7	6·19	5·86
9	6·74	5·74
23	6·62	5·03
31	6·02	5·35
32	5·00	4·22
38	5·63	5·22
41	5·73	5·44
49	5·39	4·46

*Weight at autopsy or after 12 months on trial.

Gross observations at necropsy

The animals that died experienced hair loss from all parts of the body. The skin was dry and flakey, while the hair follicles about the face became accentuated. Loss of eyelashes and swelling of the upper eyelids along with facial oedema and petechiae were present in all animals. There were also large haemorrhagic areas around the nares, on the gums and on the surfaces of the buccal cavity (Fig. 1). In addition, there were petechial haemorrhages over the entire surface of the body. Subcutaneous oedema was particularly prominent in the lower abdominal region and inner surfaces of the thighs. Marked irregularities in the growth of the toenails and fingernails (Fig. 2) and gangrenous necrosis of the distal phalanges were also recorded.

Ascites was prominent in all of the animals. There was marked distension of the intrahepatic biliary system, with oedema and dilatation (up to 1 cm in diameter) of the common bile duct (Fig. 3). Patency of bile ducts was demonstrated by the flow of bile through the ampulla of Vater and by careful dissection of the intrahepatic ducts. The lymph nodes throughout the abdominal cavity had undergone atrophy. In addition to the pale appearance of all abdominal organs, there were foci of haemorrhage in the adrenals, pancreas, liver, endometrium, serosal and mucosal surfaces of the gastro-intestinal tract and the urinary bladder.

In the thoracic cavity, the lungs exhibited focal haemorrhage in all lobes (Fig. 4). There was bilateral ventricular dilatation, cardiac enlargement and oedema. Focal areas of haemorrhage were present in the epicardium, myocardium and endocardium. The skeletal musculature, particularly in the extremities, was oedematous, pale and discoloured by haemorrhagic foci. Extensive haemorrhage was found in the meninges, and there were isolated haemorrhagic areas in the brain parenchyma. The bone marrow (from the femur, vertebrae and sternum) had the appearance

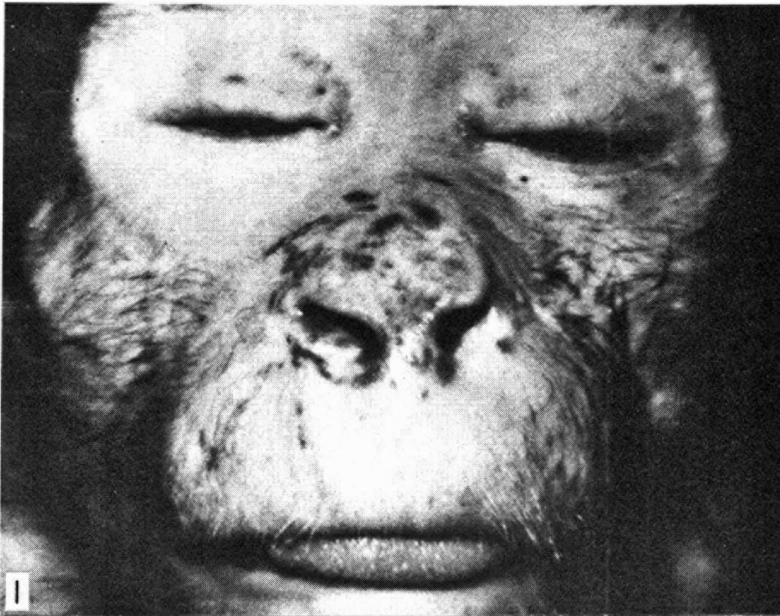
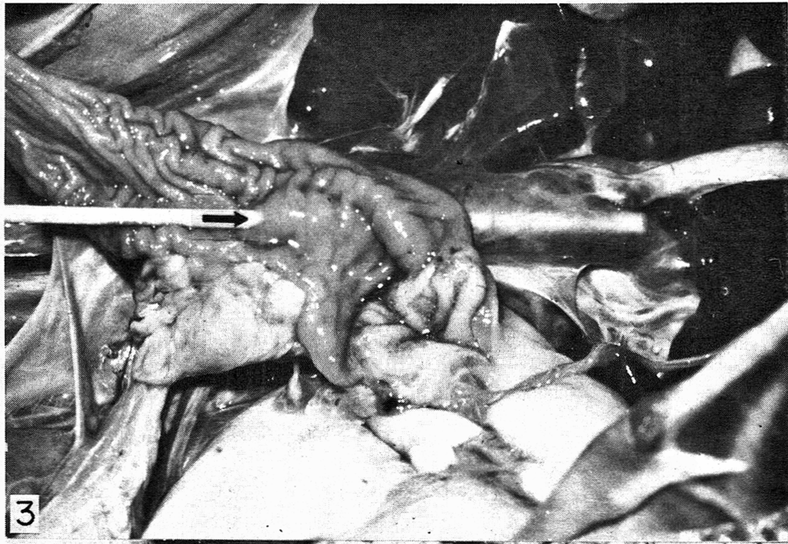
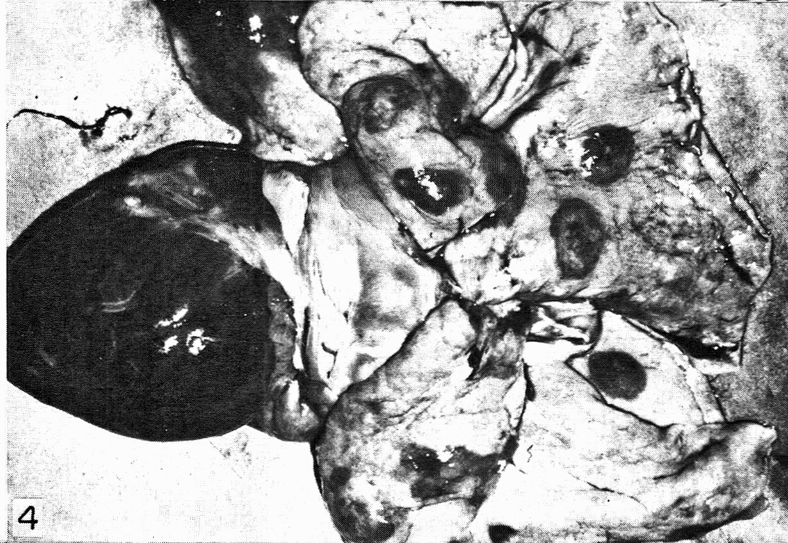


Fig. 1. Facial view of animal no. 38 which died after 9 months on a diet containing 500 ppt TCDD, showing petechial haemorrhages about the nose, absence of eyelashes and oedema of the upper eyelids and lips.

Fig. 2. Toes of animal no. 7, which died after 9 months on a diet containing 500 ppt TCDD, showing marked thickening and irregular growth of the toenails and swelling of the terminal phalanges. In the more severely affected animals, there was gangrenous necrosis of the terminal phalanges (see inset).





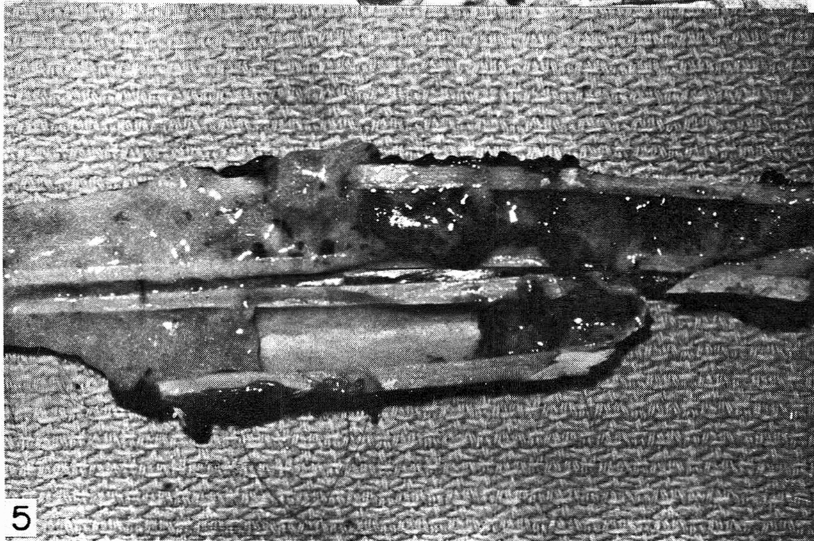


Fig. 3. In animal no. 9, which died after receiving TCDD over 9 months, a probe (arrowed) was introduced through the ampulla of Vater to demonstrate oedema and dilatation in the common bile ducts.

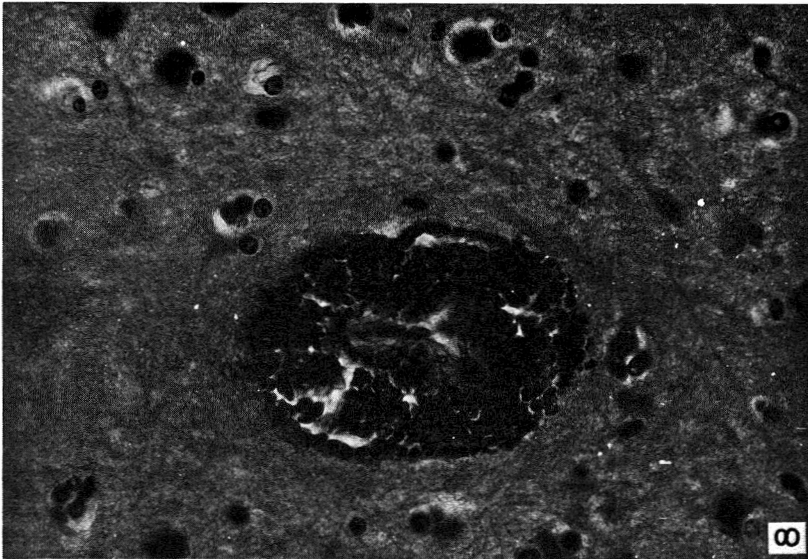
Fig. 4. Lungs and heart of animal no. 49, which died after 35 wk on a diet containing 500 ppt TCDD, showing multiple haematomas in the lobes of the lung adjacent to apparently normal parenchyma, and an enlarged heart of globoid appearance.

Fig. 5. Sample of femoral bone marrow from animal no. 49, which died 1 wk after receiving a diet containing 500 ppt TCDD for 9 months, showing the fatty appearance of the marrow and focal haemorrhages in the darker areas.

Fig. 6. Bone marrow from animal no. 32, which died with severe pancytopenia after ingesting 11·2 μg TCDD, showing small foci of darker cells dispersed among the lucent fat cells and consisting (inset) of small lymphoid-like cells with round basophilic nuclei and scant cytoplasm. Haematoxylin and eosin ×12 (inset ×104).

Fig. 7. Section of a mesenteric lymph node from animal no. 49, which died after receiving a diet containing 500 ppt TCDD for 36 wk, showing the hypocellularity of the medullary cords and, in the sinuses, widely dispersed cells which are mainly small lymphocytes and large vacuolated cells with eccentrically located nuclei. Haematoxylin and eosin ×104.

Fig. 8. Section of cerebrum from animal no. 38 which died after 9 months on diet containing 500 ppt TCDD, showing in the cortex extensive haemorrhage limited to the Virchow-Robin space around the blood vessels. Haematoxylin and eosin ×106.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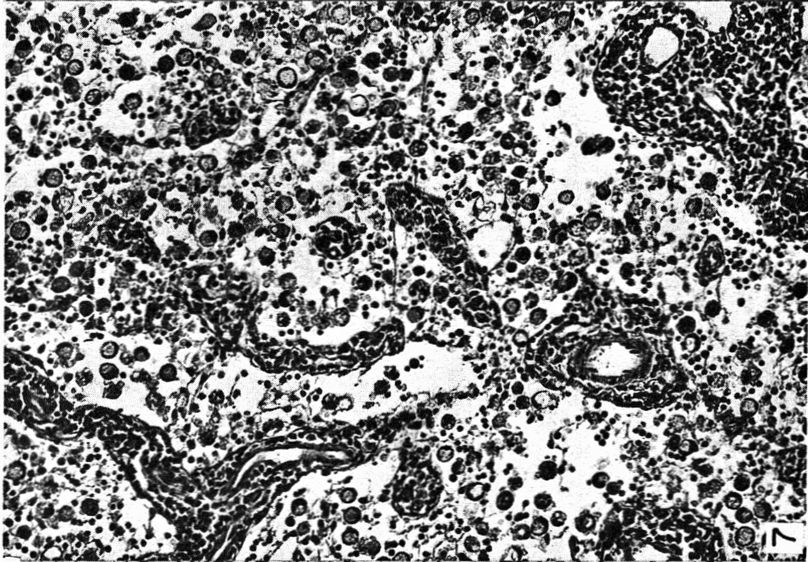
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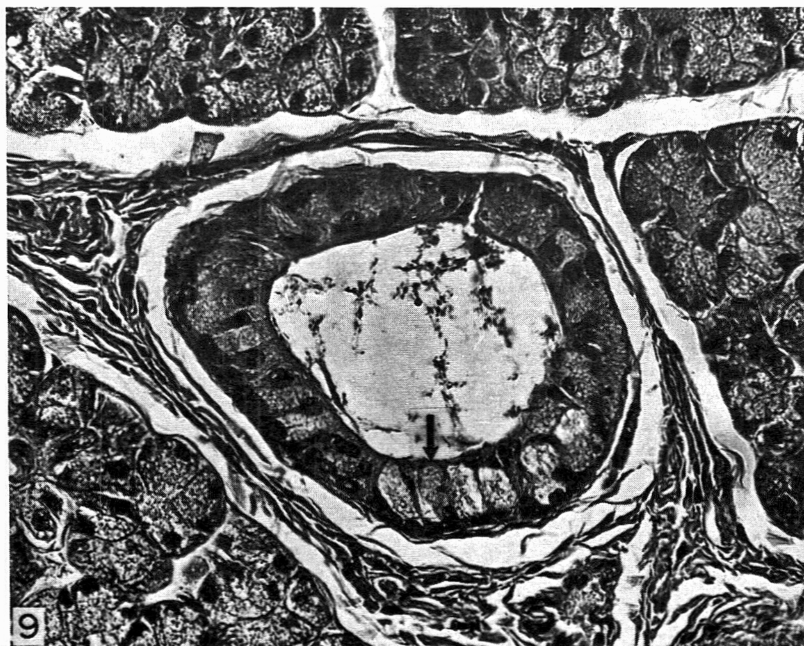
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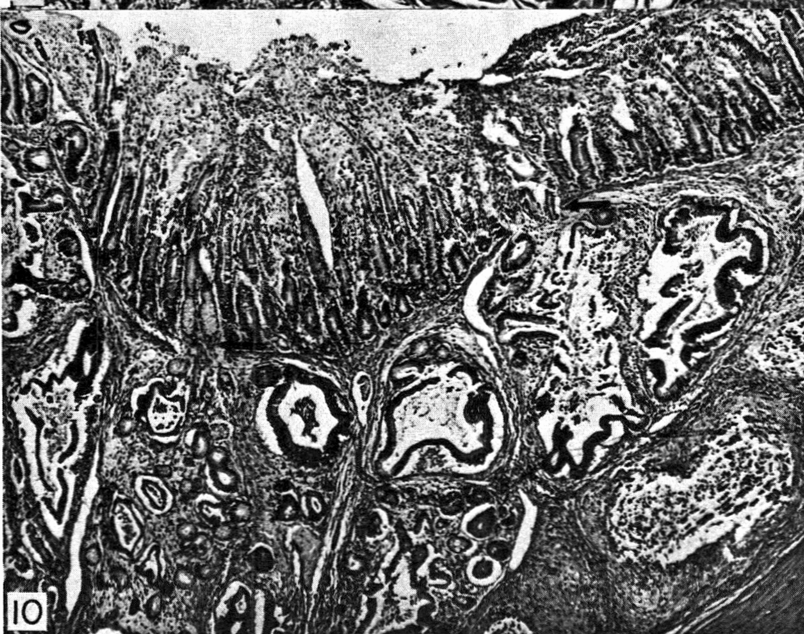
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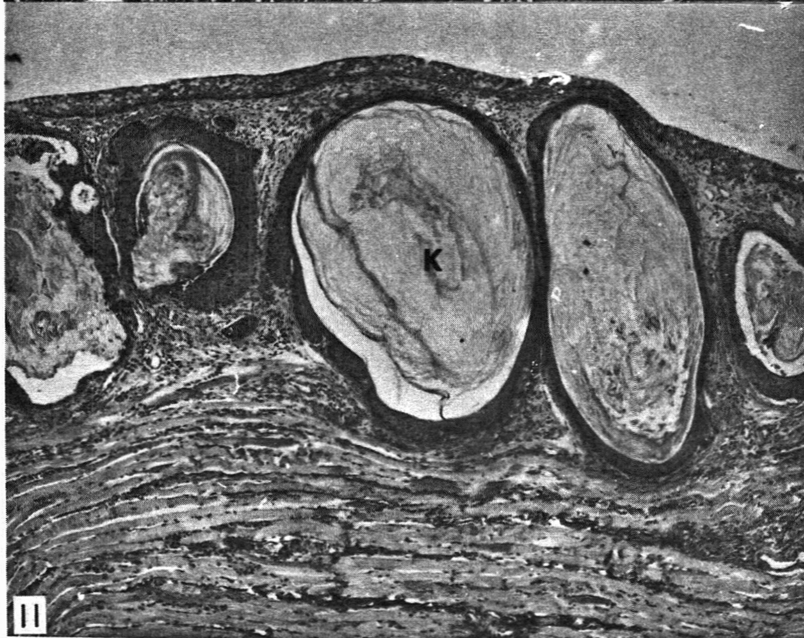
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Fig. 9. Mucus-secreting cells (arrowed) in the ductal epithelium of the salivary glands from animal no. 9. Haematoxylin and eosin × 104.

Fig. 10. Hypertrophy, hyperplasia and metaplasia of the mucus-secreting cells in the gastric mucosa of animal no. 32, typical of all monkeys exposed to TCDD, with penetration of the muscularis mucosae by the epithelium (arrowed) and the development of large areas of mucus-secreting glands in the submucosa. Haematoxylin and eosin × 12.

Fig. 11. Keratinization in the Meibomian glands of the eyelids (K) occurring as an aftermath of TCDD exposure in the monkeys, with squamous metaplasia in the epithelial cells surviving around the periphery of the gland. Haematoxylin and eosin × 12.

of fatty tissue in which there were focal areas of haemorrhage (Fig. 5).

Microscopic observations

The major microscopic changes may be separated into three categories: degeneration or atrophy of the bone marrow and lymphopoietic tissues, haemorrhage, and changes related to cellular hypertrophy, hyperplasia and metaplasia.

The bone marrow displayed a decided hypocellularity. The haematopoietic cells of the marrow were replaced primarily by fat cells with focal areas in which cells that were lymphoid in appearance predominated (Fig. 6). There was also a conspicuous reduction of discernible erythroid or myeloid stem cells as well as of megakaryocytes in the marrow. The lymph nodes throughout the body were hypocellular. In addition to the absence of any distinct lymphoid germinal centres, the cortical lymphocytes were sparse and the medullary cords were narrow or inconspicuous. Cellular debris and large vacuolated cells predominated in the sinuses (Fig. 7). The spleen, also, was devoid of any distinct lymphoid germinal centres and the small lymphocytes were widely dispersed.

The second major category of microscopic changes was related to the haemorrhage that occurred in tissues throughout the body. Well-circumscribed focal areas of haemorrhage in the heart, lungs, liver, adrenals, pancreas and skeletal muscle consisted of collections of intact red blood cells that partially disrupted the architectural pattern of the affected tissue. The acute nature of the haemorrhage was shown by the lack of tissue necrosis and an absence of reactive cells. Petechial areas were prominent in the dermis of the skin, submucosa of the urinary bladder and epithelium of the alimentary tract. Haemorrhages of the endometrium and meninges were more diffuse in their distribution. Those in the brain were limited to the Virchow-Robin spaces surrounding the blood vessels (Fig. 8). The haemorrhage in the bone marrow varied considerably, being diffuse in some areas and focal in others.

Considerable modification in the morphological features of epithelium occurred in the TCDD-treated animals. Metaplastic changes characterized by numerous mucus-secreting cells were present in the ductal epithelium of the salivary glands (Fig. 9), bile ducts and pancreatic ducts. Similar changes also occurred in the bronchial epithelium and in the palpebral conjunctivae. In addition to the metaplasia, the epithelium of the bile ducts and palpebral conjunctivae developed considerable hypertrophy and hyperplasia. Squamous metaplasia of the sebaceous glands occurred more extensively in the skin of the face and to a lesser extent in other areas.

Hypertrophy, hyperplasia and metaplasia were observed in the gastric epithelium where the parietal and zymogenic cells were replaced by those that secreted mucus. In addition to the thickened mucosal lining, many of the epithelial cells invaded the submucosa through interruptions in the muscularis mucosa (Fig. 10). Such ectopic epithelium assumed many different patterns. Some isolated cells encompassed large mucinous cysts, while others were arranged in sheets or formed acinar structures. Considerable oedematous fluid usually surrounded the epithelium. Inflammatory cells with a predominance of neutrophils were seen in close proximity to ruptured submucosal mucinous cysts. Rupture of such cysts produced the ulcers that were found in the gastric mucosa. Hypertrophy and hyperplasia were also apparent in the transitional epithelium throughout the urinary tract.

In addition to squamous metaplasia in the epithelium of the sebaceous glands, there was hyperkeratosis of the skin with keratinization of the hair follicles and the adjacent sebaceous glands. The latter alterations were particularly prominent in the Meibomian glands of the eyelids (Fig. 11). The pronounced thickening of the fingernails and toenails was considered to be secondary to excessive keratin production.

In the heart, petechial haemorrhages were prominent in the atria and in the tips of the papillary muscles. Additionally, foci of subendothelial and pericardial haemorrhage were frequently found in the ventricles. In two of the five hearts the erythrocytes were haemolysed, so that haem pigments and haemosiderin could be identified by Perls' stain in the intracellular spaces. Intra- and intercellular oedema led to separation of the muscle fibres and myofibrils.

DISCUSSION

The data presented in this report indicate that profound cellular alterations are induced in many tissues following the ingestion of minute concentrations of TCDD by primates for 9 months. Most notable are the effects of TCDD on the haematopoietic system. As exposure time lengthens, the severity of the cellular deterioration in bone marrow and lymphoid tissue becomes widespread. Terminally the animals develop severe pancytopenia. The haematological data presented here agree quite well with the previous studies conducted in this laboratory, where mixtures of dioxins, including TCDD, were fed to nonhuman primates (Allen & Carstens, 1967). In the previous investigation the monkeys also developed anaemia and leucopenia prior to death. Similar haematological changes have been recorded in rats given a single dose of 50 μg TCDD/kg body weight (Van Miller & Allen.

1977). In addition to the depletion of circulating lymphocytes, atrophy of the thymus, spleen and lymph nodes has been observed in several species (Vos, Moore & Zinkl, 1973). These changes may be related to the immune suppression which has been recorded in TCDD-exposed animals. Sublethal doses of TCDD have been shown to suppress cell-mediated and humoral immunity in guinea-pigs (Vos *et al.* 1973).

There are other complications that arise as a result of the decrease in haematopoiesis. The widespread haemorrhage that was observed in the monkeys prior to death corresponded to the decrease in blood platelets. It is logical to assume that the haemorrhage was associated at least in part with the altered clotting capability of the blood. The ventricular dilatation and myocardial hypertrophy in the TCDD-exposed monkeys may be related in part to the reduction of circulating erythrocytes and subsequent increase in the cardiac workload.

In this experiment it was shown that ingestion of 2–3 μg TCDD/kg body weight over a 9-month period was capable of producing in several organs cellular destruction or alteration sufficiently great to cause death in over 50% of the non-human primates. It is of interest that the LD_{50} of monkeys exposed to a single oral dose of TCDD is 50–70 μg/kg body weight (McConnell, Moore & Dalgard, 1977) with death occurring within 45 days. There are some major differences in the changes that occur in monkeys acutely and chronically exposed to TCDD. While death was associated with severe pancytopenia in the chronically exposed animals, haematological changes were not a constant feature of those acutely exposed. In addition, widespread haemorrhage occurred only in the chronically exposed animals. Changes that followed both acute and chronic exposure were loss of hair and nails, keratinization of the Meibomian glands and hair follicles, hyperplasia of the transitional epithelium in the renal pelvis and of the mucosal epithelium of the stomach and bile duct, a decrease in serum albumin and an increase in SGPT.

The morphological changes that have been recorded in monkeys indicate that TCDD is capable of suppressing haematopoiesis and inducing cellular alterations in epidermal appendages and mucosal epithelium of the biliary, pancreatic, intestinal and urinary tract. After exposure to TCDD, the cell population in the bone marrow and lymphoid elements underwent degeneration and necrosis. Other less specialized cells such as the bile-duct epithelium and mucous cells of the stomach were injured less severely. However, as a result of injury the latter cells showed a tendency to compensate by undergoing hypertrophy and/or hyperplasia. Some of the injured cells also reverted to more primitive forms that were capable of surviving in a less desirable environment. This was seen in the sebaceous glands where the secretory and ductal epithelium reverted to squamous cells.

The observations made on the monkeys used in this experiment indicate that there are areas that need special attention in animals, including man, exposed to TCDD. Perhaps of foremost importance are the alterations that occur in the haematopoietic tissue. Anaemia, thrombocytopenia, and leucopenia were the most debilitating changes in the primates. The altered lymphopoiesis could be associated with immune suppression. The possibility of reproductive abnormalities also exists. Altered levels of serum progesterone and oestradiol associated with difficulties in conception and early abortions have been observed in female monkeys exposed to low levels of TCDD (Allen, Barsotti & Van Miller, 1977), and testicular atrophy has developed in male monkeys given small amounts of dioxins (Allen & Carstens, 1967). In addition, the widespread hypertrophy, hyperplasia and metaplasia that occurred in the epithelium of monkeys exposed to TCDD, and a greater frequency of tumours in rats (Van Miller & Allen, 1977) suggests that the compound has carcinogenic potential.

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REFERENCES

Allen, J. R. & Carstens, L. A. (1967). Light and electron microscopic observations in *Macaca mulatta* monkeys fed toxic fat. *Am. J. vet. Res.* **28**, 1513.

Allen, J. R., Barsotti, D. A. & Van Miller, J. P. (1977). Pathological alterations in primates resulting from low level tetrachlorodibenzo-*p*-dioxin exposure. Proc. Intl Symp. Environ. Pollutants, Storrs, CT, 1–3 June 1977. In press.

Carter, C. D., Kimbrough, R. D., Liddle, J. A., Cline, R. E., Zack, M. M. & Barthel, W. F. (1975). Tetrachlorodibenzodioxin: an accidental poisoning episode in horse arenas. *Science, N.Y.* **188**, 738.

McConnell, E. E., Moore, J. A. & Dalgard, D. W. (1977). Toxicity of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) in rhesus monkeys: a single oral dose. *Toxic. appl. Pharmac.* In press.

Rose, H. R. & Rose, S. P. R. (1972). Chemical spraying as reported by refugees from South Vietnam. *Science, N.Y.* **177**, 710.

Tung, T. T. (1973). Le cancer primaire du foie au Viet-nam. *Chirurg.* **99**, 427.

Van Miller, J. P. & Allen, J. R. (1977). Chronic toxicity of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin in rats. *Fedn. Proc. Fedn. Am. Socs exp. Biol.* **36**, 396.

Vos, J. G., Moore, J. A. & Zinkl, J. G. (1973). Effect of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin on the immune system of laboratory animals. *Envir. Hlth Perspect.* no. 5, 149.

Fd Cosmet. Toxicol. Vol. 15. pp. 411–418. Pergamon Press 1977. Printed in Great Britain

MYCOTOXIC DISEASES PRODUCED IN MICE BY SPECIES OF THE *ASPERGILLUS OCHRACEUS* GROUP

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Abstract—Twenty-three isolates (identified below by Northern Regional Research Laboratory culture numbers) comprising eight species of the *Aspergillus ochraceus* group were examined for toxicity in Swiss albino mice fed for 3 wk on diets containing rice cultures of the isolates mixed at 50% concentrations with a commercial purified diet. The histopathological changes produced and a capacity to produce ochratoxin A, penicillic acid or both mycotoxins were the criteria used to assign the isolates to one of five groups. Group I (*A. auricomus* NRRL nos 387 and 388, *A. petrakii* 404, 416 and 4369, *A. elegans* 4850 and *A. alliaceus* 1237) included some strains that produced penicillic acid but none produced ochratoxin A and none caused a significant incidence of lesions or deaths. Group II isolates (*A. sclerotiorum* 415, 1598 and 4901) did not produce ochratoxin A, but two produced penicillic acid and all caused a 100% death rate, principally with lesions of multifocal hepatic necrosis and fatty change. In group III (*A. alliaceus* 315 and 4181), each isolate produced ochratoxin A and all the treated mice died. Renal lesions were consistent with those described for ochratoxicosis A. Group IV isolates (*A. sclerotiorum* 5166 and 5170) produced both ochratoxin A and penicillic acid and killed over half of the treated mice. The main hepatic and renal lesions in these mice were necrotizing cholangitis, multifocal hepatic necrosis and degeneration and necrosis of the renal convoluted tubules. The group V isolates (*A. melleus* 386 and 5103, *A. ostianus* 420 and 3524 and *A. ochraceus* 398, 399, 400, 402 and 410, as well as an isolate of *Penicillium viridicatum*, Purdue University Strain 66-68-2) did not produce ochratoxin A, but four produced penicillic acid and all caused hepatic and renal lesions identical to those observed with group IV isolates. The hepatic and renal changes observed in the mouse following treatment with either the group IV or group V isolates were similar to those described previously for an isolate of *A. ochraceus* and for several Indiana isolates of *P. viridicatum*.

INTRODUCTION

Products of cultures of an isolate of *Aspergillus ochraceus* were toxigenic when fed to male and female mice (Zimmermann, Carlton & Tuite, 1976), producing necrosis of the epithelium of the bile ducts accompanied by periductal oedema, infiltration of inflammatory cells, periductal fibrosis and disseminated focal necrosis of hepatic cells. Renal lesions included biliary casts within the convoluted tubules and collecting ducts and tubular necrosis. These changes were identical to those described in mice fed cultural products of Indiana isolates of *Penicillium viridicatum* (Budiarso, Carlton & Tuite, 1971; Carlton, Tuite & Mislivec, 1968).

The similarity of the pathological changes produced in the mouse by cultural products of *A. ochraceus* and *P. viridicatum* suggests that the toxin(s) responsible for the observed mycotoxicoses may be similar, if not identical. Other isolates belonging to the *A. ochraceus* group may also produce such toxin(s). This report describes mycotoxin production and mycotoxicoses produced in mice by isolates of eight species of the *A. ochraceus* group.

EXPERIMENTAL

Fungal isolates. Five isolates of *A. sclerotiorum*, three of *A. alliaceus*, two of *A. auricomus*, two of *A. melleus*, five of *A. ochraceus*, two of *A. ostianus*, one of *A. elegans* and three of *A. petrakii* were available for study, all from the collection of the Northern Regional Research Laboratory, Peoria, Ill. Rice cultures were prepared for each isolate, as previously described (Zimmermann *et al.* 1976), and then ground for mixing in 50% concentrations with a purified commercial diet. Each isolate was examined for its capacity to produce penicillic acid and ochratoxin A in the rice cultures prepared for animal feeding and to produce ochratoxin A production in cultures grown on YES broth.

YES cultures and extraction. Aliquots of 100 ml YES medium (2% yeast extract, 15% sucrose) in 500-ml flasks were inoculated with spore suspensions harvested from potato–dextrose agar slants (Ciegler, 1972). The culture liquor from three flasks was filtered through 24·0 cm 2V fluted filter paper, 50 ml of the filtrate being adjusted to pH 2·5 with 1 N-HCl (Ferreira. 1968) and then extracted three times with

chloroform in a separating funnel. The chloroform from the three extractions was combined and then evaporated to yield a dried residue. Thin-layer chromatography (TLC) was performed on silica-gel GHR plates using a mixture of toluene–ethyl acetate–formic acid (5:4:1, by vol.) as the solvent system. The dried extract from each isolate was dissolved in 1 ml chloroform and 10 μl of this solution was applied to the chromatographic plate together with 1- and 10-μl samples of an ochratoxin A standard (ochratoxin A, obtained from Makor Chemicals, Jerusalem, Israel) in a concentration of 100 μg/ml.

Rice culture and extraction. In the preparation of a rice culture, 128 ml H_2O was added to 280 g rice in 2-litre flasks and autoclaved for 45 min at 121°C. The rice was inoculated with spores and incubated at 24°C for 2 wk. The rice from three identical flasks was combined and three 50-g samples were blended separately with chloroform (250 ml) in a Waring blender for 3 min and then filtered in a Buchner funnel using no. 2 filter paper. The original material was reblended in 250 ml chloroform and filtered. The two chloroform extracts were combined and dried over sodium sulphate for 15 min, the chloroform was decanted and the residue was dried (Ciegler, 1972).

The TLC method used was a modification of the method described by Ciegler (1972). After addition of 1 ml chloroform to the residue from the rice extraction, 10-μl aliquots were spotted on silica-gel GHR plates developed first with ethyl ether to remove interfering substances and then with toluene–ethyl acetate–formic acid (5:4:1, by vol.). The ochratoxin A standard (1 and 10 μl) was also spotted.

In examinations for penicillic acid production, 50-g samples of rice cultures of each isolate were placed in 500-ml glass-stoppered flasks and 25 ml 0·5 N-phosphorous acid and 250 ml chloroform were added. The flasks were shaken mechanically for 30 min and the contents were filtered through fluted paper with approximately 10 g diatomaceous earth. The filtered extracts were placed on chromatography columns and fractionated using the method of Wilson, Tabor & Trucksess (1975). The residues obtained after chromatography were diluted with 1·0 ml chloroform and 30-μl aliquots were spotted onto silica G-25 HR plates. The plates were first developed in ethyl ether (Trucksess, 1974) for approximately 25 min and were then dried and developed in toluene–ethyl acetate–formic acid (5:4:1, by vol.). The plates were dried again and exposed to ammonia fumes for 15 min and the resultant fluorescent spots were examined under long-wave ultraviolet light.

Toxicity studies. The experimental diets containing rice cultures of the fungal isolates and a purified commercial diet in equal quantities were fed to groups of 15 male and female Swiss albino mice. The mice were weighed at weekly intervals and provided with feed and water *ad lib.* Autopsies were performed on mice that died, on those killed when moribund and on the survivors killed after a 3-wk period of feeding. The liver, kidneys, stomach, duodenum, small intestine, pancreas, spleen and mesenteric lymph nodes were fixed in 10% buffered formalin, processed for paraffin sectioning, and stained with haematoxylin and eosin for histopathological examination.

RESULTS

The types of histopathological lesions produced in mice by the test diets and the ability of the cultures to produce ochratoxin A, penicillic acid or both mycotoxins were used to assign the isolates to one of five groups.

Group I

Mice fed diets containing 50% levels of rice cultures of *A. auricomus* NRRL nos 387 and 388, *A. petrakii* nos 404, 416 and 4369, *A. elegans* no. 4850 and *A. alliaceus* no. 1237 showed no signs of toxicosis during the 3-wk feeding period and body-weight gains averaged 8 g (Table 1). The isolates were ochratoxin A-negative, but *A. auricomus* nos 387 and 388 and *A. petrakii* no. 416 were producers of penicillic acid (Table 2). None of the mice fed isolates in this group showed gross or histological changes.

Group II

All mice fed diets containing rice cultures of *A. sclerotiorum* nos 415, 1598 and 4901 died during the feeding period (Table 1). Each isolate was negative for ochratoxin A but isolates 415 and 4901 produced penicillic acid (Table 2). As early as day 5 of feeding, the mice were lethargic and huddled together. By day 7, some mice had posterior weakness and roughened hair and most were depressed, some severely. Gross changes were similar for all isolates, consisting predominantly of clotted blood within the gastric lumen, severe congestion of the gastro-intestinal serosa and liver, and pale and swollen kidneys (Table 3).

The main microscopic change produced by *A. sclerotiorum* isolates 415 and 1598 was multifocal hepatic necrosis (Table 4). No specific hepatic structure was consistently included in the necrotizing process and most of the necrotic foci were located paracentrally or adjacent to central veins rather than periportally. Nuclear pleomorphism and megalocytosis were observed in hepatocytes adjacent to the necrotic foci. Mice fed *A. sclerotiorum* isolate 4901 had severe centrilobular hepatic fatty change, but no necrosis. Renal lesions consisted of degeneration and necrosis of the convoluted tubules, but were not observed with isolate 4901 (Table 5).

Group III

A. alliaceus isolates 315, 1237, and 4181, and *A. petrakii* 4369 were reported by Hesseltine, Vandegraft, Fennell, Smith & Shotwell (1972) to produce ochratoxin A when grown on various grain substrates (Table 2). Production of ochratoxin A was demonstrated for *A. alliaceus* nos 315 and 4181, but not for *A. alliaceus* no. 1237 or *A. petrakii* no. 4369 and the latter pair were therefore assigned to group I. None of these four cultures was positive for penicillic acid (Table 2). The death rates in the treated mice correlated directly with ochratoxin A production, being 100% in groups fed cultures of *A. alliaceus* isolates 4181 and 315. Macroscopic renal changes observed with the 4181 and 315 isolates included pale and slightly swollen kidneys (Table 3), but only isolate 315 produced macroscopic hepatic alterations (diffuse subcapsular paleness and accentuation of lobules).

Microscopic hepatic lesions noted in one mouse fed

Table 1. *Body weights and deaths in groups of mice fed rice cultures of isolates of the* A. ochraceus *group*

Group	Species	NRRL no.	Mean body weight at wk				No. of deaths*
			0	1	2	3	
I	*A. alliaceus*	1237	13	16	17	19	0
	A. auricomus	387	13	16	18	21	1
		388	13	16	17	21	0
	A. petrakii	404	14	17	17	20	0
		416	13	16	18	20	1
		4369	13	18	18	23	0
	A. elegans	4850	14	16	18	23	0
II	*A. sclerotiorum*	415	14	—	—	—	15
		1598	13	11	—	—	15
		4901	13	11	—	—	15
III	*A. alliaceus*	315	12	9	—	—	15
		4181	12	10	—	—	15
IV	*A. sclerotiorum*	5166	13	13	11	11	8
		5170	13	13	12	11	8
V	*A. melleus*	386	13	11	—	—	15
		5103	13	—	—	—	15
	A. ostianus	420	13	14	13	13	15
		3524	14	12	11	—	15
	A. ochraceus	398	12	—	—	—	15
		399	11	12	13	16	7
		400	13	10	—	—	15
		402	15	16	16	19	2
		410	14	11	—	—	15
	P. viridicatum	66-68-2†	14	12	10	—	15

*By end of wk 3, from groups of 15 mice.
†Code number of culture collection at Purdue University.

Table 2. *Ochratoxin and penicillic acid production by isolates of the* A. ochraceus *group*

Group	Species	NRRL no.	Ochratoxin A production (ppm)			Penicillic acid production (ppm)	
			H*	C†	T‡	T‡	C†
I	*A. alliaceus*	1237	388	NT	—	—	NT
	A. auricomus	387	—	NT	—	30	NT
		388	—	NT	—	0·3	NT
	A. petrakii	404	—	NT	—	—	NT
		416	—	NT	—	3	NT
		4369	0·7	NT	—	—	NT
	A. elegans	4850	—	NT	—	—	NT
II	*A. sclerotiorum*	415	—	NT	—	+	NT
		1598	—	NT	—	—	NT
		4901	—	—	—	100	2600
III	*A. alliaceus*	315	1000	+	50	—	—
		4181	2	+	20	—	—
IV	*A. sclerotiorum*	5166	0·300	NT	20	+	NT
		5170	0·126	+	50	1–2	1180
V	*A. melleus*	386	—	+§	—	6	1860
		5103	—	NT	—	34	NT
	A. ostianus	420	—	NT	—	—	NT
		3524	—	NT	—	30	NT
	A. ochraceus	398	+§	—	—	—	—
		399	—	+§	—	—	—
		400	—	—	—	—	1330
		402	—	—	—	20/30	602
		410	—	—	—	—	—
	P. viridicatum	66-68-2	NT	NT	—	—‖	NT

— = Negative + = Positive NT = Not tested
*Reported by Hesseltine *et al.* (1972).
†Reported by Ciegler (1972).
‡As determined by J. Tuite (this study).
§In small quantity.
‖Reported by Carlton *et al.* 1968.

Table 3. *Gross lesions in mice fed rice cultures of isolates of the* A. ochraceus *group*

				Hepatic lesions					Renal lesions	
Group	Species	Code No.	Icterus	Congestion	Paleness	Accentuated lobulation	Necrosis/ haemorrhage	Cystic bile ducts	Green discoloration	Paleness/ swelling
I	*A. alliaceus*	1237	—	0	0	0	0	0	0	0
	A. auricomus	387	—	0	0	0	0	0	0	0
		388	—	0	0	0	0	0	0	0
	A. petrakii	404	—	0	0	0	0	0	0	0
		416	—	0	0	0	0	0	0	0
		4369	—	0	0	0	0	0	0	0
	A. elegans	4850	—	0	0	0	0	0	0	0
II	*A. sclerotiorum*	415	—	2	0	0	0	0	0	7
		1598	—	3	0	0	0	0	0	3
		4901	—	2	0	0	0	0	0	3
III	*A. alliaceus*	315	—	1	0	0	0	0	0	3
		4181	—	0	0	0	0	0	3	0
IV	*A. sclerotiorum*	5166	—	0	0	0	1	2	1	6
		5170	—	3	0	0	1	0	1	2
V	*A. melleus*	386	6	4	0	0	6	1	5	3
		5103	0	1	1	1	0	0	0	4
	A. ostianus	420	6	4	3	1	5	2	1	6
		3524	6	1	1	1	5	2	4	6
	A. ochraceus	398	0	0	1	1	2	4	0	2
		399	2	2	1	0	0	0	3	3
		400	0	0	1	2	3	0	0	3
		402	2	3	0	0	1	0	1	2
		410	0	3	0	0	0	0	1	5
	P. viridicatum	66-68-2	6	3	2	0	2	1	3	3

The incidence of lesions is expressed in terms of the numbers of mice affected in test groups of 15 animals.

Table 4. *Histopathological changes in the livers of mice fed rice cultures of isolates of the* A. ochraceus *group*

Group	Species	Code no.	Pericholangitis	Necrotizing cholangitis	Multifocal necrosis	Extrahepatic-duct necrosis
I	*A. alliaceus*	1237	0	0	0	0
	A. auricomus	387	0	0	0	0
		388	0	0	0	0
	A. petrakii	404	0	0	0	0
		416	0	0	0	0
		4369	0	0	0	0
	A. elegans	4850	0	0	0	0
II	*A. sclerotiorum*	415	0	0	4	0
		1598	0	0	2	0
		4901	0	0	0	0
III	*A. alliaceus*	315	0	0	1	0
		4181	0	0	0	0
IV	*A. sclerotiorum*	5166	6	5	4	2
		5170	3	4	4	1
V	*A. melleus*	386	7	15	7	5
		5103	12	13	7	5
	A. ostianus	420	8	13	13	7
		3524	11	15	7	10
	A. ochraceus	398	8	13	9	3
		399	4	15	3	2
		400	9	15	10	3
		402	5	15	3	—
		410	11	15	8	2
	P. viridicatum	66-68-2	9	15	14	8

The incidence of lesions is expressed in terms of the numbers of mice affected in test groups of 15.

Table 5. *Histopathological changes in the kidneys of mice fed rice cultures of isolates of the* A. ochraceus *group*

			Incidence of		
			Tubular casts		
Group	Species	Code no.	Hyaline	Biliary	Tubular necrosis
I	*A. alliaceus*	1237	0	0	0
	A. auricomus	387	0	0	0
		388	0	0	0
	A. petrakii	404	0	0	0
		416	0	0	0
		4369	0	0	0
	A. elegans	4850	0	0	0
II	*A. sclerotiorum*	415	0	0	6
		1598	0	0	3
		4901	0	0	0
III	*A. alliaceus*	315	0	0	3
		4181	0	0	4
IV	*A. sclerotiorum*	5166	1	1	1
		5170	0	3	0
V	*A. melleus*	386	2	2	6
		5103	0	0	6
	A. ostianus	420	1	2	5
		3524	3	3	3
	A. ochraceus	398	0	3	3
		399	0	4	0
		400	1	0	6
		402	1	2	0
		410	1	1	7
	P. viridicatum	66-68-2	0	3	7

The incidence of lesions is expressed in terms of the numbers of mice affected in test groups of 15.

A. alliaceus isolate 315 consisted of several disseminated foci of hepatocellular necrosis (Table 4), while mice fed isolate 4181 developed hepatic lesions including hepatocellular megalocytosis and mild fatty change. Necrosis and degeneration of the epithelium of the convoluted tubules were observed in mice fed *A. alliaceus* isolates 4181 and 315 (Table 5). Alterations included nuclear pyknosis, karyorrhexis, and haryolysis and separation of the epithelial cells from the basement membrane. Some cellular debris was present within the lumina of the convoluted tubules, but no casts were observed. Necrosis was seen occasionally in the renal epithelial cells of Henle's loop and in the collecting ducts. One mouse fed *A. alliaceus* isolate 315 had necrosis of the superficial gastric mucosa.

Group IV

A. sclerotiorum isolates 5166 and 5170 were reported by Hesseltine *et al.* (1972) to produce ochratoxin A, and they produced both ochratoxin A and penicillic acid when grown on rice (Table 2). By the end of the 3-wk feeding period, 50% of the animals in each group had died and the survivors had lost weight (Table 1). Jaundice was seen in two mice after 10 days on the diet containing *A. sclerotiorum* isolate 5170.

Macroscopic alterations induced by both isolates of *A. sclerotiorum* included pale and swollen kidneys, hepatic congestion and accentuation of the hepatic lobules (Table 3). Discrete green-to-yellow foci were observed consistently in the mice fed either isolate and were distributed throughout all lobes of the liver. Cystic dilatation of extrahepatic bile ducts was noted only in mice fed isolate 5166. Green discoloration of the kidneys was noted in mice fed either isolate.

Microscopic hepatic lesions in mice fed *A. sclerotiorum* nos 5166 and 5170 included acute or subacute pericholangitis, necrotizing cholangitis, multifocal hepatocellular necrosis and necrosis of the extrahepatic ducts (Table 4). The pericholangitis was usually minimal, consisting of plasma cells or lymphocytes admixed with fewer neutrophils. Early changes in the biliary ducts included flattening of the epithelium and periductal oedema. Focal degeneration and necrosis of the epithelium followed, culminating in a severe necrotizing cholangitis. In some livers, fibroblasts had proliferated about the bile ducts and early regenerative changes were apparent, consisting of hypertrophy and hyperplasia of the biliary epithelium. Multifocal hepatocellular necrosis was produced by each isolate and was observed in association with necrotic bile ducts. A severe cholecystitis was observed in mice fed either isolate of *A. sclerotiorum*. Focal or diffuse necrosis of the mucosal epithelium was accompanied by necrosis of the gall-bladder wall in some mice. Several gall bladders that were free of mucosal necrosis had eosinophilic debris admixed with inflammatory leucocytes in their lumina. Extrahepatic bile-duct necrosis was observed in some mice fed either isolate. Frequently, periductal oedema with moderate numbers of mononuclear inflammatory cells was observed. Eosinophilic proteinaceous plugs were occasionally present within periductal lymphatics.

Renal lesions were mild in mice fed *A. sclerotiorum* isolates 5166 and 5170 and consisted of mild cortical tubular necrosis and/or the presence of biliary pigment or hyaline casts within intact tubules (Table 5).

Group V

All of the remaining ten fungal isolates (*A. ochraceus* nos 398, 399, 400, 402 and 410, *A. melleus* nos 386 and 5103, *A. ostianus* nos 420 and 3524 and *P. viridicatum* no. 66-68-2) were negative for ochratoxin A production, but *A. melleus* isolates 386 and 5103, *A. ostianus* no. 3524 and *A. ochraceus* no. 402 produced varying amounts of penicillic acid (Table 2). Most of the mice fed these isolates died during the first 2 wk of the study and by the end of wk 3 the only survivors were in the groups fed *A. ochraceus* nos 399 and 402. The earliest change indicative of toxicosis in the treated mice was a roughened coat. Subsequently, the mice developed arched backs and tense abdomens, became lethargic and died. Some mice that survived the first 8 days of feeding became icteric and some had green urine (Table 3). The principal gross hepatic change was the development of pinpoint yellow-green to red and grey foci distributed throughout all lobes. In some livers, large portions of individual lobes were affected. Cystic and dilated extrahepatic bile ducts were noted, but occurred infrequently. Other hepatic observations included paleness, marked congestion, and accentuation of the lobules. The latter changes were prominent in livers that did not have other lesions (Table 3). Renal changes varied from a marked greenish discoloration to kidneys that were pale and swollen (Table 3).

Hepatic lesions produced by all the nine Aspergillus isolates (three species) and by the *P. viridicatum* isolate were histologically similar. Early bile-duct changes included individual cell vacuolation and flattening and periductal oedema. Pericholangitis was prominent, with mononuclear leucocytes and a few neutrophils. Hepatocytes around the triads with cholangitis showed megalocytosis and cytoplasmic vacuolation. Severe necrosis of the bile-duct cells was accompanied by necrosis of the other portal structures as well as of adjacent hepatocytes. Periportal hepatocellular necrosis appeared to expand from the affected triads with the formation of larger foci of necrosis around the eosinophilic remnants of the portal structures. Within the severely affected lobes, coalescence of adjacent necrotic foci produced large areas of necrosis. Dystrophic mineralization and suppurative hepatitis frequently developed within these larger areas of necrosis (Table 4). In some livers, large areas of necrosis extended from the portal triads to the capsular surface and were suggestive of infarction. When the hepatic capsule was breached, a focal acute suppurative peritonitis developed.

Periductal fibrosis was observed in the livers of mice fed isolates of *A. melleus* no. 386 and *P. viridicatum* no. 66-68-2 and bile-duct proliferation was noted in mice fed *A. ochraceus* no. 402, *A. ostianus* no. 420, *A. melleus* 5103 and *P. viridicatum* no. 66-68-2. These types of bile-duct change were found in mice dying after 1 wk of test feeding.

Extrahepatic bile ducts often had lesions similar to those found in the intrahepatic bile ducts, including periductal oedema, cuffs of mixed inflammatory cells and focal or diffuse cholangitis. The lymphatics within the fibrous connective tissue surrounding the extrahe-

patic ducts often contained plugs of eosinophilic proteinaceous material. Necrosis of extrahepatic bile ducts within the pancreatic interlobular connective-tissue stroma was observed with the isolates *A. melleus* no. 386, *A. ostianus* nos 420 and 3524 and *A. ochraceus* nos 398 and 400 and was accompanied by pancreatitis in some mice.

Cholecystitis was consistently observed except in the mice fed *A. ochraceus* no. 402. This group had the lowest number of deaths and fewer and milder hepatic lesions With most isolates the cholecystitis was severe and necrosis involved both the mucosal wall and adjacent fibrous connective tissue.

Lesions were observed in the kidneys of mice fed each of the ten fungal isolates and consisted of dilatation and necrosis of convoluted tubules and the presence of bile pigment and hyaline casts within the lumina of the tubules.

DISCUSSION

Mycotoxins produced by members of the *A. ochraceus* group include ochratoxin A and penicillic acid. These compounds were produced singly or in combination by some of the isolates examined in these experiments. Penicillic acid, a substituted lactone, is produced by *A. ochraceus* (Karow, Woodruff & Foster, 1944; Natori, Sakaki, Kurata, Udagawa, Ichinoe, Saito & Umeda, 1970; Udagawa, Ichinoe & Kurata, 1970), by *A. melleus* and *A. quercinus* (Burton, 1949) and by *A. ostianus*, *A. sulphureus* and members of the genus Penicillium (Kawasaki, Oki, Umeda & Saito, 1972). The elaboration of penicillic acid was associated with several isolates that produced hepatic lesions, with isolates that produced no lesions, and with isolates that evoked a high death rate but produced no specific histopathological changes.

Penicillic acid has been reported to induce in mice sinusoidal ectasia, histiocytic cell infiltration and generalized necrosis of hepatocytes (Ciegler, 1972). Hepatic necrosis was not observed consistently in mice fed the isolates from group II that produced penicillic acid, and the type of hepatic necrosis, when it did occur, was neither generalized nor accompanied by an infiltration of histiocytes. *A. sclerotiorum* no. 4901 yielded the largest amount of penicillic acid and the significant histopathological change observed was severe centrilobular fatty change. Several isolates in group V produced penicillic acid, but no correlation could be established regarding any increase in deaths between those isolates that produced the toxin and those that did not. In fact the isolate associated with the fewest deaths, *A. ochraceus* no. 402, produced a considerable amount of penicillic acid. A specific histological lesion was observed in all of the groups of mice fed isolates from group V, with a necrotizing cholangitis as the principal change. Such a lesion is not consistent with the histological picture described for penicillic acid toxicosis in the mouse. Penicillic acid was also produced by several isolates from group I but these caused neither deaths nor any microscopic change. In view of the available histological data relating to penicillic acid toxicity in the mouse, and the lack of association between toxin production, a high death rate and the development of lesions, the possibility that penicillic acid was a significant factor in the deaths and lesions that occurred among mice fed isolates of groups I, II and V seems doubtful.

The group III isolates *A. alliaceus* nos 315 and 4181 produced ochratoxin A, and all the animals fed these isolates had died by wk 2 of feeding. The principal pathological alterations were degeneration and necrosis of the renal convoluted tubules, disseminated hepatocellular necrosis, megalocytosis and fatty change. Ochratoxin A toxicosis in the mouse has been described as a haemorrhagic disorder with widespread congestion of abdominal organs, gastritis and enteritis resulting from impairment of the vascular endothelium or of one of the factors in haemostasis (Galtier, More & Bodin, 1974). The changes observed in the mice in our studies were more consistent with those described for ochratoxicosis A in rats, namely necrosis of the convoluted tubules and hepatocytes (Purchase, 1968). The toxic effects of ochratoxin A in the mouse appear to vary with physiological status, strain and possible metabolic factors.

A. sclerotiorum isolates 5166 and 5170 in group IV each produced both ochratoxin A and penicillic acid. The hepatic lesions induced by these isolates, however, were not like those described for either ochratoxin A or penicillic acid, but instead were similar to the changes previously described in the livers of mice fed an isolate of *A. ochraceus* (Zimmermann *et al.* 1976) or any of the isolates included in group V.

All ten isolates in group V produced similar hepatic and renal changes, including a necrotizing cholangitis with subsequent multifocal hepatic necrosis, and degeneration and necrosis of the renal convoluted tubules. This pathological spectrum has been observed previously with Indiana isolates of *P. viridicatum* (Budiarso *et al.* 1971; Carlton *et al.* 1968) and *A. ochraceus* (Zimmermann *et al.* 1976), and again no specific mycotoxins could be identified as being responsible for the observed histological changes. Although some of the isolates in group V produced penicillic acid, not all did so, and those that did included those associated with the highest and the lowest numbers of deaths. Penicillic acid, therefore, was not considered to be the mycotoxin responsible for the disease that developed in mice fed these isolates.

In addition to ochratoxin A and penicillic acid, other metabolites produced by specis of *A. ochraceus* include ochracin (mellein), hydroxyaspergillic acid and neohydroxyaspergillic acid (Natori *et al.* 1970). Kojic acid was produced by an isolate of *A. alliaceus* (Gill-Carey, 1949) and other members of the genus Aspergillus (Parrish, Wiley, Simmons & Long, 1966). The isolates included in this study were not examined for aspergillic acid production, but these acids were eliminated as possible causative metabolites because of the absence of any reports associating them with hepatotoxicity (Bush, Dickinson, Ward & Avery, 1945). Mellein and 4-hydroxymellein are both isocoumarin derivatives and have been isolated from *A. oniki* and *A. ochraceus* including an ochratoxin A-producing strain of the latter (Cole, Moore, Davis, Kirksey & Diener, 1971; Sasaki, Kaneko, Oshita, Takamatsu, Asao & Yokotsuka, 1970). Both compounds are related structurally to the ochratoxins (van der Merwe, Steyn & Fourie, 1965), and Moore, Davis & Diener (1972) proposed that mellein probably had biological properties similar to those of ochratoxin

A. Kojic acid administered to mice caused prostration, laboured breathing, hind limb paresis and convulsions followed by death (Jennings & Williams, 1945; Werch, Oester & Friedemann, 1957). Similar clinical signs were not observed in the study reported here.

The occurrence of a consistent spectrum of lesions centering about the biliary ducts in mice treated with the group IV and group V isolates, including that of *P. viridicatum*, suggested that the toxin(s) produced by these members of the *A. ochraceus* group were similar to those produced by *P. viridicatum*. It may be that these isolates produce xanthomegnin and viomellein, as these two metabolites of *P. viridicatum* (Stack, Eppley, Dreifuss & Pohland, 1977), and of *A. sulphureus* and *A. melleus* (Durley, MacMillan, Simpson, Glen & Turner, 1975) have been reported to produce hepatic lesions identical to those induced by cultural products of *P. viridicatum* (Carlton, Stack & Eppley, 1976).

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REFERENCES

Budiarso, I. T., Carlton, W. W. & Tuite, J. (1971). Investigation of dose, age and duration of administration on the hepatorenal damage induced in mice by cultural products of *Penicillium viridicatum*. *Toxic. appl. Pharmac.* **20,** 357.

Burton, H. S. (1949). Antibiotics from Penicillia. *Br. J. exp. Path.* **30,** 151.

Bush, M. T., Dickinson, H. L., Ward, C. B. & Avery, R. C. (1945). Antibiotic substances active against *M. tuberculosis*. *J. Pharmac. exp. Ther.* **85,** 237.

Carlton, W. W., Stack, M. E. & Eppley, R. M. (1976). Hepatic alterations produced in mice by xanthomegnin and viomellein, metabolites of *Penicillium viridicatum*. *Toxic. appl. Pharmac.* **38,** 455.

Carlton, W. W., Tuite, J. & Mislivec, P. (1968). Investigations of the toxic effects in mice of certain species of *Penicillium*. *Toxic. appl. Pharmac.* **13,** 372.

Ciegler, A. (1972). Bioproduction of ochratoxin A and penicillic acid by members of the *Aspergillus ochraceus* group. *Can. J. Microbiol.* **18,** 631.

Cole, R. J., Moore, J. H., Davis, N. D., Kirksey, J. W. & Diener, U. L. (1971). 4-Hydroxymellein: A new metabolite of *Aspergillus ochraceus*. *J. agric. Fd Chem.* **19,** 909.

Durley, R. C., MacMillan, J., Simpson, T. J., Glen, A. T. & Turner, W. B. (1975). Fungal products XIII. Xanthomegnin, viomellein, rubrosulphin and viopurpurin, pigments from *Aspergillus sulphureus* and *Aspergillus melleus*. *J. chem. Soc. Perkin Trans.* **I,** 163.

Ferreira, N. P. (1968). The effect of amino acids on the production of ochratoxin A in chemically defined media. *Antonie van Leeuwenhoek* **34,** 433.

Galtier, P., More, J. & Bodin, G. (1974). Toxins of *Aspergillus ochraceus* Wilhelm. III—Acute toxicity of ochratoxin A in adult rats and mice. *Ann. Rech. Vet.* **5,** 233.

Gill-Carey, D. (1949). The nature of some antibiotics from Aspergilli. *Br. J. exp. Path.* **30,** 119.

Hesseltine, C. W., Vandegraft, E. E., Fennell, D. I., Smith, M. L. & Shotwell, O. L. (1972). Aspergilli as ochratoxin producers. *Mycologia* **64,** 539.

Jennings, M. A. & Williams, T. I. (1945). Production of kojic acid by *A. effusus* Tiraboschi. *Nature, Lond.* **155,** 302.

Karow, E. O., Woodruff, H. B. & Foster, J. W. (1944). Penicillic acid from *A. ochraceus, P. thomii, P. suavolens*. *Archs Biochem.* **5,** 279.

Kawasaki, I. L., Oki, T., Umeda, M. & Saito, M. (1972). Cytotoxic effect of penicillic acid and patulin on HeLa cells. *Jap. J. exp. Med.* **42,** 327.

Moore, J. H., Davis, N. D. & Diener, U. L. (1972). Mellein and 4-hydroxymellein production by *Aspergillus ochraceus* Wilhelm. *Appl. Microbiol.* **23,** 1067.

Natori, S., Sakaki, S., Kurata, H., Udagawa, S., Ichinoe, M., Saito, M. & Umeda, M. (1970). Chemical and cytotoxicity survey on the production of ochratoxins and penicillic acid by *Aspergillus ochraceus* Wilhelm. *Chem. Pharm. Bull., Tokyo* **183,** 2259.

Parrish, F. W., Wiley, B. J., Simmons, E. G. & Long, L., Jr. (1966). Production of aflatoxins and kojic acid by species of *Aspergillus* and *Penicillium*. *Appl. Microbiol.* **14,** 139.

Purchase, I. F. H. & Theron, J. J. (1968). The acute toxicity of ochratoxin A to rats. *Fd Cosmet. Toxicol.* **6,** 479.

Sasaki, M., Kaneko, Y., Oshita, K., Takamatsu, H., Asao, Y. & Yokotsuka, T. (1970). Studies on the compounds produced by molds. Part VII. Isolation of isocoumarin compounds. *Agric. biol. Chem.* **34,** 1296.

Stack, M. E., Eppley, R. M., Dreifuss, P. A. & Pohland, A. E. (1977). Isolation and identification of xanthomegnin, viomellein, rubrosulphin and viopurpurin as metabolites of *Penicillium viridicatum*. *Appl. envir. Microbiol.* **33,** 351.

Trucksess, M. (1974). Observation on the thin layer chromatographic development of corn extracts with ethyl ether. *J. Ass. off. analyt. Chem.* **57,** 1220.

Udagawa, S. I., Ichinoe, M. & Kurata, H. (1970). Occurrence and distribution of mycotoxin producers in Japanese foods. Proc. First US–Japan Conference on Toxic Microorganisms, Honolulu. US Dept of Interior and UJNR Panel on Toxic Microorganisms, Washington, D.C.

van der Merwe, K. J., Steyn, P. S. & Fourie, L. (1965). Mycotoxins. Part II. The constitution of ochratoxins A, B and C, metabolites of *Aspergillus ochraceus* Wilh. *J. chem. Soc.* p. 7083.

Werch, S. C., Oester, Y. T. & Friedemann, T. E. (1957). Kojic acid—a convulsant. *Science, N.Y.* **126,** 450.

Wilson, D. M., Tabor, W. H. & Trucksess, M. W. (1975). Screening method for the detection of aflatoxin, ochratoxin, zearalenone, penicillic acid and citrinin. *J. Ass. off. analyt. Chem.* **59,** 125.

Zimmermann, J. L., Carlton, W. W. & Tuite, J. (1976). Mycotoxicosis produced in mice by cultural products of an isolate of *Aspergillus ochraceus*. *Fd Cosmet. Toxicol.* **14,** 571.

Fd Cosmet. Toxicol. Vol. 15. pp. 419–422. Pergamon Press 1977. Printed in Great Britain

INHIBITION OF YEAST ALCOHOL DEHYDROGENASE BY DEHYDRORETRONECINE

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Abstract—The interaction of dehydroretronecine, a hepatocarcinogenic metabolite of the pyrrolizidine alkaloid monocrotaline, with yeast alcohol dehydrogenase (ADH), cysteine and bovine serum albumin (BSA) was studied. Dehydroretronecine inhibited ADH with an inhibition constant (K_i) of $3{\cdot}38 \times 10^{-2}$ M at pH 7·5 and 25°C. No inhibitory effect was observed when excess cysteine was added to the assay system. The interaction of dehydroretronecine with cysteine was demonstrated by spectrophotometric titration. Titration of dehydroretronecine alone at 240 nm showed an apparent pK value of 5·0, whereas titration of dehydroretronecine in the presence of excess cysteine resulted in an increase of pK from 5·0 to 8·5. Equilibrium dialysis data indicated appreciable binding of dehydroretronecine to BSA at pH 4·2 and 6°C.

INTRODUCTION

Plants containing the pyrrolizidine alkaloids are distributed worldwide, and include many species in a wide variety of genera including Senecio, Crotalaria, Heliotropium, Trichodesma and Amsinckia (Kingsbury, 1964). Livestock poisoning by consumption of pyrrolizidine alkaloid-containing plants has recently become a major problem in the Pacific northwest and other parts of the United States and the world (Deinzer, Thomson, Burgett & Isaacson, 1977). Consumption by man of foods and herbal medicines contaminated with pyrrolizidine alkaloids has resulted in acute hepatic veno-occlusive disease which progresses to liver cirrhosis (McLean, 1970). In addition, animal experiments have shown that certain pyrrolizidine alkaloids are carcinogenic (Allen, Hsu & Carstens, 1975; Harris & Chen, 1970; Svoboda & Reddy, 1972), mutagenic (Cook & Holt, 1966; Culvenor, Downing, Edgar & Jago, 1969) and teratogenic (Green & Christie, 1961).

Dehydroretronecine is the major detectable toxic metabolite of the pyrrolizidine alkaloid, monocrotaline (Hsu, Allen & Chesney, 1973). This pyrrole is responsible for ulceration and atrophy of the gastric mucosa, rhabdomyosarcomas (Allen *et al.* 1975) and megalohepatocytosis. The discovery of the oncogenic nature of dehydroretronecine prompted investigations of the pyrrole's interaction with cellular macromolecules *in vivo* and *in vitro* (Hsu, Robertson & Allen, 1976; Hsu, Robertson, Shumaker & Allen, 1975). Their data suggest a direct correlation of the levels of dehydroretronecine binding to cellular macromolecules with the lesions that develop in affected organs. Hayashi & Lalich (1968) have shown that injections of either mercaptoethylamine or cysteine will prolong survival and improve the growth rate of rats that receive monocrotaline parenterally. Recently, protective effects of dietary cysteine have been reported in rats affected by pyrrolizidine alkaloid poisoning (Buckmaster, Cheeke & Shull, 1976). This paper presents an account of the binding of dehydroretronecine to cysteine and bovine serum albumin (BSA) and the inhibitory effect of dehydroretronecine on a thiol enzyme, yeast alcohol dehydrogenase (ADH).

EXPERIMENTAL

Enzyme assay. ADH derived from yeast and nicotinamide adenine dinucleotide (NAD) were purchased from Sigma Chemical Co. (St. Louis, Mo.). The activity of yeast ADH was determined by the method of Vallee & Hoch (1955) by measuring the rate of increase in absorbancy at 340 nm, 1 unit being defined as the amount of enzyme that reduced 1 μmol NAD/min at 25°C under the conditions specified. ADH concentration was determined spectrophotometrically at 280 nm using an extinction coefficient, $E^{0{\cdot}1\%}_{1\,cm}$, of 12·6 (Hayes & Valick, 1954) and a molecular weight of 151,000 (Kagi & Vallee, 1960).

Inhibition studies. The inhibitory effect of dehydroretronecine on ADH activity was determined by analysis of the initial velocity of the enzyme-mediated reaction in the presence and absence of dehydroretronecine in the reaction mixture. All data were treated by the methods of Lineweaver & Burk (1934) and Hunter & Downs (1945). The ability of cysteine to block the inhibition was determined by adding freshly prepared cysteine solution to the reaction mixture containing dehydroretronecine, substrate and ADH.

Spectral analyses and spectrophotometric titrations. Absorption data were obtained by measuring the appropriate solutions in a Beckman Model DU spectrophotometer with a light path of 1 cm at room temperature. Spectrophotometric titrations were carried out in a Radiometer automatic titrator with a Radiometer Titrigraph type SBR2 and SBU1 syringe burette as described by Chu (1971). Samples were withdrawn from the titration vessel at appropriate pH values, and optical density was measured at 240 nm.

Preparation of dehydroretronecine and [^3H]dehydroretronecine. Monocrotaline was extracted from the seed of *Crotalaria spectabilis* by the procedure of Koekemoer & Warren (1950). Dehydroretronecine was prepared from the parent alkaloid, monocrotaline, according to the method of Culvenor, Edgar, Smith & Tweeddale (1970). Dehydroretronecine tritiated at C-7 and C-9 (specific activity 20 μCi/mg) was synthesized by a new procedure (M.-T. S. Hsia. unpublished data 1977) based on the reduction of 1-formyl-7β-hydroxy-6,7-dihydro-5*H*-pyrrolizine with NaB^3H_4 (New England Nuclear, Boston, Mass; > 100 mCi/nmol).

Equilibrium dialysis. Equilibrium dialysis was carried out at 6°C according to the method of Rosenberg & Klotz (1960). Detailed procedures were essentially those of Chu (1974), except that tritiated dehydroretronecine was used to calculate the free dehydroretronecine concentration. Radioactivity data were obtained by counting in 10 ml of a solution of Insta-Gel universal liquid scintillation cocktail (Packard Instrument Co., Downers Grove, IL) using a Beckman Model 335 liquid scintillation spectrometer. A molecular weight of 65.000 (Spahr & Edsall. 1964) was used for the calculation of the molar concentration of BSA.

RESULTS

Effect of dehydroretronecine on enzyme activity

The inhibitory effect of dehydroretronecine on ADH activity is clearly demonstrated in Fig. 1, which also shows the effect of varying the concentration of dehydroretronecine on ADH activity at a constant concentration of substrate. To examine the type of inhibition produced, the substrate concentration was varied in the presence and absence of dehydroretronecine ($7{\cdot}84 \times 10^{-5}$ M). The results, plotted according to the method of Lineweaver & Burk (1934). are shown in Fig. 2. The plot indicates that dehydroretronecine has a mixed-type (competitive and non-competitive) effect on ADH. The inhibition constant (K_i) was found to be $3{\cdot}38 \times 10^{-2}$ M under the conditions studied.

Blocking effect of cysteine on enzyme inhibition

Table 1 shows the results of adding cysteine to the enzyme assay system at zero time in the presence of dehydroretronecine. The inhibitory effect of dehydroretronecine on ADH activity was blocked completely by the addition of cysteine at a concentration 8–15 times greater than that of dehydroretronecine.

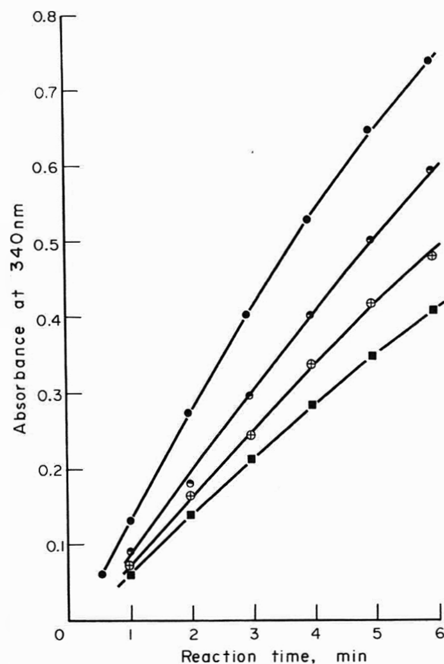

Fig. 1. Effect of dehydroretronecine on the enzyme activity of ADH. The reaction was carried out in a pH 7·5 phosphate buffer at 25°C in the absence of dehydroretronecine (●) and in the presence of $7{\cdot}84 \times 10^{-5}$ M (⊖), $2{\cdot}61 \times 10^{-4}$ M(⊕) and $3{\cdot}27 \times 10^{-4}$ M (■) DR. The concentrations of enzyme, ethanol and NAD were $3{\cdot}05 \times 10^{-6}$ M, 2·0 M and $2{\cdot}5 \times 10^{-4}$ M, respectively.

Spectrophotometric titrations

Since dehydroretronecine showed the maximum difference between absorbance in acidic and basic conditions at 240 nm, spectrophotometric titrations were carried out at this wavelength. The titration of dehydroretronecine alone showed an apparent pK of 5·0 (estimated from the half-titration pH) as shown in Fig. 3 (curve d). In the presence of cysteine the pK value shifted from 5·0 to 8·5 (Fig. 3, curve b). Binding between dehydroretronecine and cysteine was evident from the drastic shift in position of the titration curve.

Table 1. *Effect of cysteine on the inhibition of ADH activity by dehydroretronecine*

Incubation mixture*	Relative enzyme activity (%)
Control	100
Cysteine†	100
Dehydroretronecine‡	75
Dehydroretronecine‡ + cysteine†	100
Dehydroretronecine§	62
Dehydroretronecine§ + cysteine†	100

*The assay conditions were the same as those described in Fig. 2.
†Freshly prepared solution of cysteine was added at a final concentration of $3{\cdot}3 \times 10^{-4}$ M.
‡The dehydroretronecine concentration was $2{\cdot}18 \times 10^{-5}$ M.
§The dehydroretronecine concentration was $4{\cdot}36 \times 10^{-5}$ M.

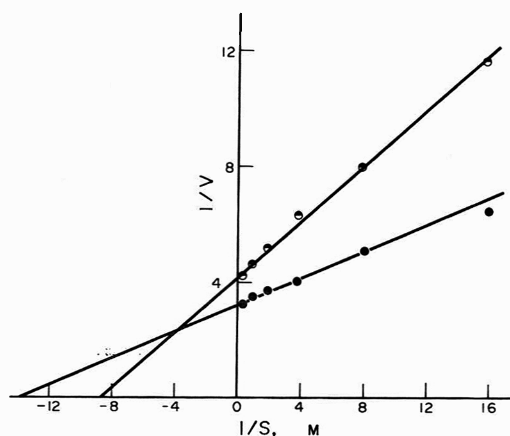

Fig. 2. Lineweaver-Burk plots for ADH; control reaction (●): reaction inhibited by $2{\cdot}61 \times 10^{-4}$ M dehydroretronecine (◓). The reaction was carried out in a pH 7·5 phosphate buffer at 25°C. The concentrations of enzyme, ethanol and NAD were $1{\cdot}52 \times 10^{-6}$ M and $2{\cdot}5 \times 10^{-4}$ M, respectively.

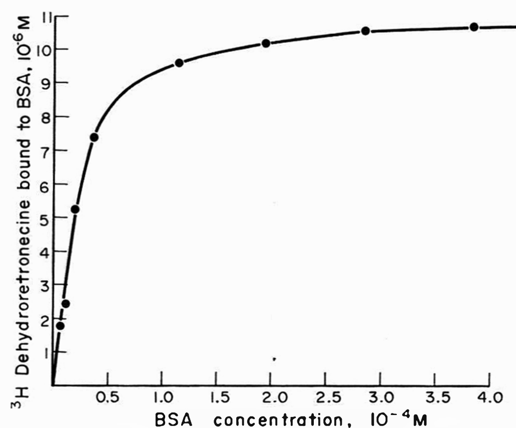

Fig. 4. Binding of [^{3}H]dehydroretronecine with BSA. The data were obtained by equilibrium dialysis at pH 4·2 and 6°C. The [^{3}H]dehydroretronecine concentration was $1{\cdot}13 \times 10^{-4}$ M; the BSA concentration varied from $7{\cdot}6 \times 10^{-6}$ to $3{\cdot}85 \times 10^{-4}$ M.

Binding of dehydroretronecine with bovine serum albumin

The binding curve of dehydroretronecine with BSA obtained from equilibrium dialysis in pH 4·2 citrate–phosphate buffer is shown in Fig. 4. The extrapolated value obtained from the curve indicates that 0·25 mol dehydroretronecine is bound by 1 mol BSA. However, there was no binding of BSA with dehydroretronecine at pH 7·0.

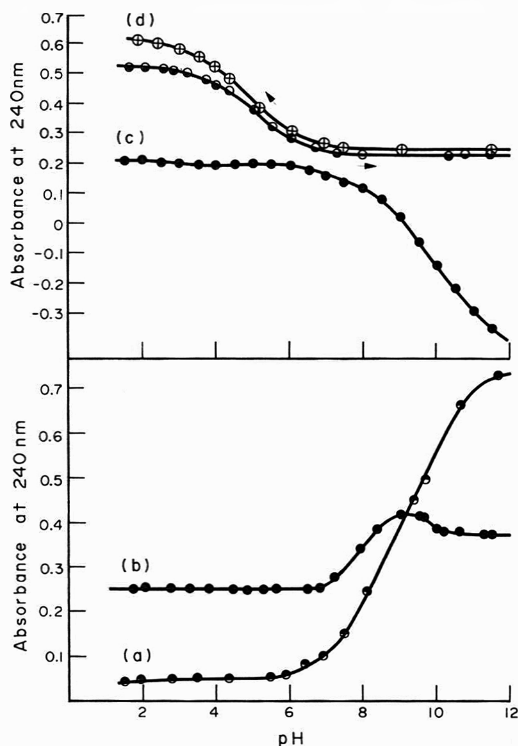

Fig. 3. Spectrophotometric titration of cysteine and the dehydroretronecine–cysteine complex: (a) titration data of cysteine alone; (b) titration data of dehydroretronecine–cysteine complex; (c) the difference between the data of curves b and a (b − a); (d) titration of free dehydroretronecine. The starting concentration of dehydroretronecine was $3{\cdot}27 \times 10^{-4}$ M.

DISCUSSION

The inhibition of ADH activity by dehydroretronecine and the binding of dehydroretronecine with cysteine and BSA have been demonstrated by spectrophotometric analyses and equilibrium dialysis. Inhibition of ADH by *p*-chloromercuribenzoate has been reported by Snodgrass, Vallee & Hoch (1960), and the inhibition was reversible on addition of excess glutathione. It is possible that the inhibitory effect of dehydroretronecine on ADH was due to the reaction of dehydroretronecine with the thiol groups in the active centre of ADH. The importance of the interaction with thiol compounds to the *in vivo* toxic action of pyrrolizidine alkaloids can be seen from the protective effect of ip-injected mercaptoethylamine and cysteine against monocrotaline intoxication in rats (Hayashi & Lalich, 1968). This is substantiated by the recent report that dietary cysteine has a protective effect on rats affected by pyrrolizidine alkaloid poisoning (Buckmaster, Cheeke & Shull, 1976). However, the protective mechanism of thiol compounds against pyrrolizidine alkaloid intoxication is obscure. Cysteine administration reduced the incidence of chromosomal breakage in Allium roots treated with monocrotaline or allied pyrrolizidine alkaloids (Avanzi, 1961). By analogy with data from radiobiology, it was suggested that cysteine protection against pyrrolizidine alkaloids was operating by lowering the intracellular oxygen tension.

As far as we are aware, the present paper contains the first report on the inhibitory effect of a reactive pyrrolizidine alkaloid metabolite, dehydroretronecine, on the activity of an enzyme (ADH) under *in vitro* conditions, and the reverse effect of cysteine on such enzyme activity. Current literature shows that the alkylation of various cell components is one probable mechanism by which dehydroretronecine produces pathological alterations, whether they be gastric ulceration, hepatocellular necrosis or rhabdomyosar-

comas. On the basis of our present results, it might be postulated that thiol compounds exert their protective effect by competing with the nucleophilic components of the cells as potential target(s) for alkylation by the pyrrole metabolite(s).

The interaction of dehydroretronecine with BSA was first studied by spectrophotometric analysis (P. Sun and F. S. Chu, unpublished data 1976). Positive absorptivity was observed at 220–300 nm when dehydroretronecine was incubated with BSA at pH 2·90, 5·22 or 7·20. However, calf-thymus DNA showed negative absorptivity under the same conditions. These results correlated with previous observations (Culvenor *et al.* 1969; Hsu *et al.* 1975) that there was a preferential binding of dehydroretronecine to proteins over other cellular components including nucleic acids. Results from equilibrium dialysis indicated no binding to BSA at pH 7·0. However, dehydroretronecine readily bound to BSA at pH 4·2. The most likely reason for this increased binding at a lower pH is increased protonation of the allylic hydroxyl group(s) in dehydroretronecine with subsequent dehydration, forming a relatively stable allylic carbonium ion which can undergo electrophilic alkylation of macromolecules. Although the significance of the interaction of dehydroretronecine with BSA at neutral pH is not known at present, BSA may serve as a transport agent for this pyrrole, thus providing a constant liberation of the toxic metabolite from the pyrrole–albumin complex to target tissue(s).

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REFERENCES

Allen, J. R., Hsu, I. C. & Carstens, L. A. (1975). Dehydroretronecine induced rhabdomyosarcomas in rats. *Cancer Res.* **35**, 997.

Avanzi, S. (1961). Chromosome breakage by pyrrolizidine alkaloids and modification of the effect by cysteine. *Caryologia* **14**, 251.

Buckmaster, G. W., Cheeke, P. R. & Shull, L. R. (1976). Pyrrolizidine alkaloid poisoning in rats: protective effects of dietary cysteine. *J. Anim. Sci.* **43**, 464.

Chu, F. S. (1971). Interaction of ochratoxin A with bovine serum albumin. *Archs Biochem. Biophys.* **147**, 359.

Chu, F. S. (1974). A comparative study of the interaction of ochratoxins with bovine serum albumin. *Biochem. Pharmac.* **23**, 1105.

Cook, L. M. & Holt, A. C. E. (1966). Mutagenic activity in *Drosophila* of two pyrrolizidine alkaloids. *J. Genet.* **59**, 273.

Culvenor, C. C. J., Downing, D. T., Edgar, J. A. & Jago, M. V. (1969). Pyrrolizidine alkaloids as alkylating and antimitotic agents. *Ann. N.Y. Acad. Sci.* **163**, 837.

Culvenor, C. C. J., Edgar, J. A., Smith, L. W. & Tweeddale, H. J. (1970). Dihydropyrrolizines. III. Preparation and reactions of derivatives related to pyrrolizidine alkaloids. *Aust. J. Chem.* **23**, 1853.

Deinzer, M. L., Thomson, P. A., Burgett, D. M. & Isaacson, D. L. (1977). Pyrrolizidine alkaloids: their occurrence in honey from tansy ragwort (*Senecio jacobaea* L.). *Science, N.Y.* **195**, 497.

Green, C. R. & Christie, G. S. (1961). Malformations in foetal rats induced by the pyrrolizidine alkaloid, heliotrine. *Br. J. exp. Path.* **42**, 369.

Harris P. N. & Chen, K. K. (1970). Development of hepatic tumors in rats following ingestion of *Senecio longilobus*. *Cancer Res.* **30**, 2881.

Hayashi, Y. & Lalich, J. J. (1968). Protective effect of mercaptoethylamine and cysteine against monocrotaline intoxication in rats. *Toxic. appl. Pharmac.* **12**, 36.

Hayes, J. E. & Valick, S. F. (1954). Yeast alcohol dehydrogenase: molecular weight, coenzyme binding, and reaction equilibria. *J. biol. Chem.* **207**, 225.

Hsu, I. C., Allen, J. R. & Chesney, C. F. (1973). Identification and toxicological effects of dehydroretronecine, a metabolite of monocrotaline. *Proc. Soc. exp. Biol. Med.* **144**, 835.

Hsu, I. C., Robertson, K. A., Shumaker, R. C. & Allen, J. R. (1975). Binding of tritiated dehydroretronecine to macromolecules. *Res. Commun. chem. Path. Pharmac.* **11**, 99.

Hsu, I. C., Robertson, K. A. & Allen, J. R. (1976). Tissue distribution, binding properties and lesions produced by dehydroretronecine in the nonhuman primate. *Chemico-Biol. Interactions* **12**, 19.

Hunter, A. & Downs, C. E. (1945). The inhibition of arginase by amino acids. *J. biol. Chem.* **157**, 427.

Kagi, J. H. R. & Vallee, B. L. (1960). The role of zinc in alcohol dehydrogenase. V. The effect of metal-binding agents on the structure of the yeast alcohol dehydrogenase molecule. *J. biol. Chem.* **235**, 3188.

Kingsbury, J. M. (1964). *Poisonous Plants of the United States and Canada*. p. 425. Prentice-Hall, Inc., New Jersey.

Koekemoer, M. J. & Warren, F. L. (1950). The *Senecio* alkaloids. VIII. The occurrence and preparation of the N-oxides. An improved method of extraction of the *Senecio* alkaloids. *J. chem. Soc.* p. 60.

Lineweaver, H. & Burk, D. (1934). The determination of enzyme dissociation constants. *J. Am. chem. Soc.* **56**, 658.

McLean, E. K. (1970). The toxic actions of pyrrolizidine (*Senecio*) alkaloids. *Pharmac. Rev.* **22**, 429.

Rosenberg, R. M. & Klotz, I. M. (1960). Dye binding methods. In *A Laboratory Manual of Analytical Methods of Protein Chemistry*. Vol. 2. Edited by P. Alexander and R. J. Block, p. 133. Pergamon Press, Oxford.

Snodgrass, P. J., Vallee, B. L. & Hoch, F. L. (1960). Effects of silver and mercurials on yeast alcohol dehydrogenase. *J. biol. Chem.* **235**, 504.

Spahr, P. F. & Edsall, J. T. (1964). Amino acid composition of human and bovine serum albumin mercaptoalbumins. *J. biol. Chem.* **239**, 850.

Svoboda, D. & Reddy, J. K. (1972). Malignant tumors in rats given lasiocarpine. *Cancer Res.* **32**, 908.

Vallee, B. L. & Hoch, F. L. (1955). Zinc, a component of yeast alcohol dehydrogenase. *Proc. natn. Acad. Sci. U.S.A.* **41**, 327.

Fd Cosmet. Toxicol. Vol. 15. pp. 423–430. Pergamon Press 1977. Printed in Great Britain

N-NITROSODIETHANOLAMINE IN COSMETICS, LOTIONS AND SHAMPOOS

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Abstract—*N*-Nitrosodiethanolamine (NDElA), a compound known to produce liver tumours in rats, was detected in widely used consumer products such as cosmetics, hand and body lotions and hair shampoos. The concentration varied from less than 1 ng/g (ppb) to 48,000 ng/g, the latter in a facial cosmetic. The source of the NDElA was presumably the nitrosation of the di- and/or triethanolamine additives. NDElA was identified by coincidence of retention time on three different high-pressure liquid chromatograph columns using an *N*-nitrosamine-specific detector. In a single case the compound eluting at the retention time of NDElA was also isolated and identified by high-resolution mass spectrometry.

INTRODUCTION

N-Nitrosamines are a group of potent chemical carcinogens (Druckrey, Preussmann, Ivankovic & Schmähl, 1967; Magee & Barnes, 1956; Magee, Montesano & Preussmann, 1976) which may be implicated in the aetiology of human cancer (Lijinsky & Epstein, 1970). Dimethylnitrosamine and *N*-nitrosopyrrolidine have been reported to be present in some foodstuffs preserved with nitrite, especially cooked bacon (Crosby, Foreman, Palframan & Sawyer, 1972; Scanlan, 1975; Sen, Seaman & Miles, 1976). More recently, *N*-nitrosamines have been identified in soil and water (Fine, Rounbehler, Rounbehler & Silvergleid, 1977b), as air pollutants (Fine, Rounbehler, Belcher & Epstein, 1976; Fine, Rounbehler, Pellizzari, Bunch, Berkley, McCrae, Bursey, Sawicki, Krost & DeMarrais, 1976) and as major impurities in some widely used herbicides (Fine, Rounbehler, Fan & Ross, 1977a).

Di- and/or triethanolamine are widely used in industry (*Merck Index*, 1976) in the manufacture of pesticides, surface-active agents, cutting fluids and as solvents for casein, shellac and dyes. In addition, triethanolamine is used as a penetrating agent for wood and paper, as well as an emulsifier in waxes, polishes and toilet goods. Lijinsky, Keefer, Conrad & Von de Bogard (1972) demonstrated that *N*-nitrosodiethanolamine (NDElA) could be formed readily by nitrosation of either di- or triethanolamine. When fed to rats, NDElA has been shown to produce liver tumours (Druckrey *et al.* 1967) with a potency about two hundred times less than that of diethylnitrosamine. Schmeltz, Abidi & Hoffman (1977) found NDElA to be present in unburned processed tobacco which had been treated with the herbicide, maleic hydrazide, formulated as the diethanolamine salt. Fan, Morrison, Rounbehler, Ross, Fine, Miles & Sen (1977) reported NDElA to be present as a major impurity in industrial cutting fluids which had been formulated with triethanolamine and sodium nitrite. Because of the apparent ease with which the secondary and tertiary ethanolamines form the *N*-nitroso derivative, some widely used consumer products that contain ethanolamines were analysed for NDElA.

EXPERIMENTAL

Materials. Consumer skin-care products, cosmetics, beauty aids and hair shampoos were purchased over the counter in the Boston area and were representative of those products that are most widely used. Triethanolamine (AR grade, J. T. Baker, Chemical Co., Phillipsburg, N.J.), sodium nitrite (ACS reagent grade; Fisher Scientific Co., Fairlawn, N.J.) and silica gel (HiFlosil support for column chromatography, 60/200 mesh, Applied Science, State College, PA), were used without further purification. Acetone, ethyl acetate, hexane, iso-octane, isopropanol, methanol, methylene chloride and tetrahydrofuran were glass-distilled and supplied by Burdick and Jackson (Muskegon, MI).

N-Nitrosodiethanolamine. NDElA was prepared according to the procedure of Druckery *et al.* (1967). The nitrosated product was purified by repeated isopropanol precipitation of unreacted sodium nitrite and by elution from a silica-gel column. NDElA is a light-yellow oil. The authenticity of the synthesized NDElA was established by mass spectrometry and proton magnetic resonance. Both low- and high-resolution mass spectra were determined with a CEC 21-110B mass spectrometer equipped with a direct sample introduction system and operated at a source temperature of 130°C, an ionizing potential of 70 eV and an ionizing current of 200 μA. Figure 1 shows the low-resolution mass spectrum of authentic

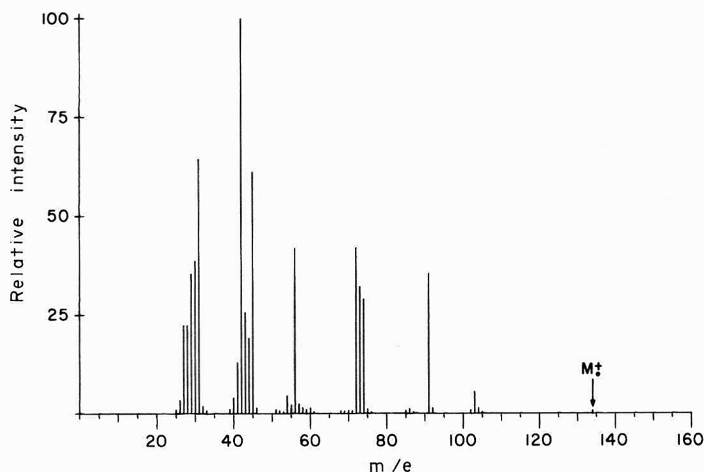

Fig. 1. Low resolution mass spectrum of authentic NDEIA, mol wt 134, shiwing a molecular ion of less than 1% relative intensity at *m/e* 134.

NDEIA and Fig. 2 shows the high-resolution mass spectrum, in the form of an element map (Biemann, Bommer & Desiderio, 1964), of the same material. The 60 MHz proton magnetic resonance spectrum was taken on acetone-d_6 (minimum 99·5 atom% D) on a Hitachi Perkin Elmer R-20B spectrometer using tetramethylsilane as internal standard and is shown in Fig. 3.

Apparatus. The high-pressure liquid chromatograph (HPLC) was constructed by connecting in sequence a high-pressure pump (Waters Associates, Milford, Mass., Model 6000A and/or Varian, Model 8500), a high pressure flow injector (Waters Associates, Model U6K), a 3 in. long Corasil II precolumn (for crude samples), an HPLC column, a collection valve (of our own design) and a Thermal Energy Analyser (TEA; Thermo Electron, TEA Model 502LC). For conventional HPLC operation, the liquid flow was from the column, through the valve and into the TEA detector. In the collection mode, conventional HPLC operation was followed until the peak eluting at the proper retention time was just seen to enter the TEA. The flow was then diverted by the valve into the collection vial.

Quantitative analysis procedure. For testing, 5 g of the product was weighed into a 250-ml conical flask and 250 mg ammonium sulphamate was added. The mixture was stirred for 1 min until all the ammonium sulphamate crystals had dissolved. Ethyl acetate (100 ml) was added, and the mixture was stirred at room temperature for 15 min using a Teflon-coated magnetic stirrer. The stirred mixture was filtered through 40 g anhydrous sodium sulphate, and washed with 50 ml ethyl acetate. The filtrate and washings were combined and passed through a 25-cm length of glass column (1 cm ID) packed with 20 g silica gel. The ethyl acetate fraction was discarded, and the column was then washed with 100 ml acetone. The acetone fraction was collected, with the column being allowed to go to dryness. The acetone was evaporated to 5–10 ml using a vacuum rotary evaporator operating at room temperature (22°C). The concentrate was filtered (Whatman No. 1) into a 25-ml Kuderna tube and the filter was washed twice with 5 ml acetone. The combined filtrate and washings were concentrated under a stream of nitrogen to 0·5–0·8 ml. The TEA–HPLC was injected with 25 μl of the final concentrate. The HPLC was operated isocratically using 40% acetone and 60% iso-octane at a flow rate of 2 ml/min.

At least two control experiments were carried out on each product shown to contain a measurable amount of NDEIA. In the first, 0·050 g triethanolamine (1%) and 0·005 g sodium nitrite (0·1%) were added to the product following the addition of ammonium sulphamate. In the second, an excess of NDEIA was added to the product to determine the efficiency of the recovery. The excess of NDEIA was ten times the amount initially found in the product.

Further control experiments were carried out with lotion L9, which contained only a trace of NDEIA. Triethanolamine, and triethanolamine in combination with sodium nitrite were added at various concentrations in order to demonstrate that no NDEIA was formed during the analytical procedure.

Isolation procedure for mass-spectrometric analysis. Samples of commercial products (100 g) were freeze-dried and then extracted for 30 min with 500 ml ethyl acetate while the mixture was stirred with a Teflon-coated magnetic stirrer. The ethyl acetate was dried by passing it through 200 g anhydrous sodium sulphate. The eluate was concentrated on a rotary evaporator to 200 ml, filtered through filter paper and loaded onto a silica column (250 × 20 mm). The column was eluted in sequence with 100 ml each of ethyl acetate, 30 and 70% acetone (in ethyl acetate) and acetone. Fractions of 50 ml were collected and concentrated to 1–5 ml on the rotary evaporator and then chromatographed on TEA–HPLC. The fractions that showed a significant NDEIA peak were pooled and taken to 0·5 ml under a stream of nitrogen. The material eluting at the retention time of NDEIA was collected from the TEA–HPLC, which was operated isocratically (40% acetone and 60% hexane) at a flow rate of 2 ml/min using a μ Porasil column (Waters Associates) with a Corasil II precolumn (Waters Associates). The collected HPLC eluate was concentrated and then chromatographed on a μ Styragel

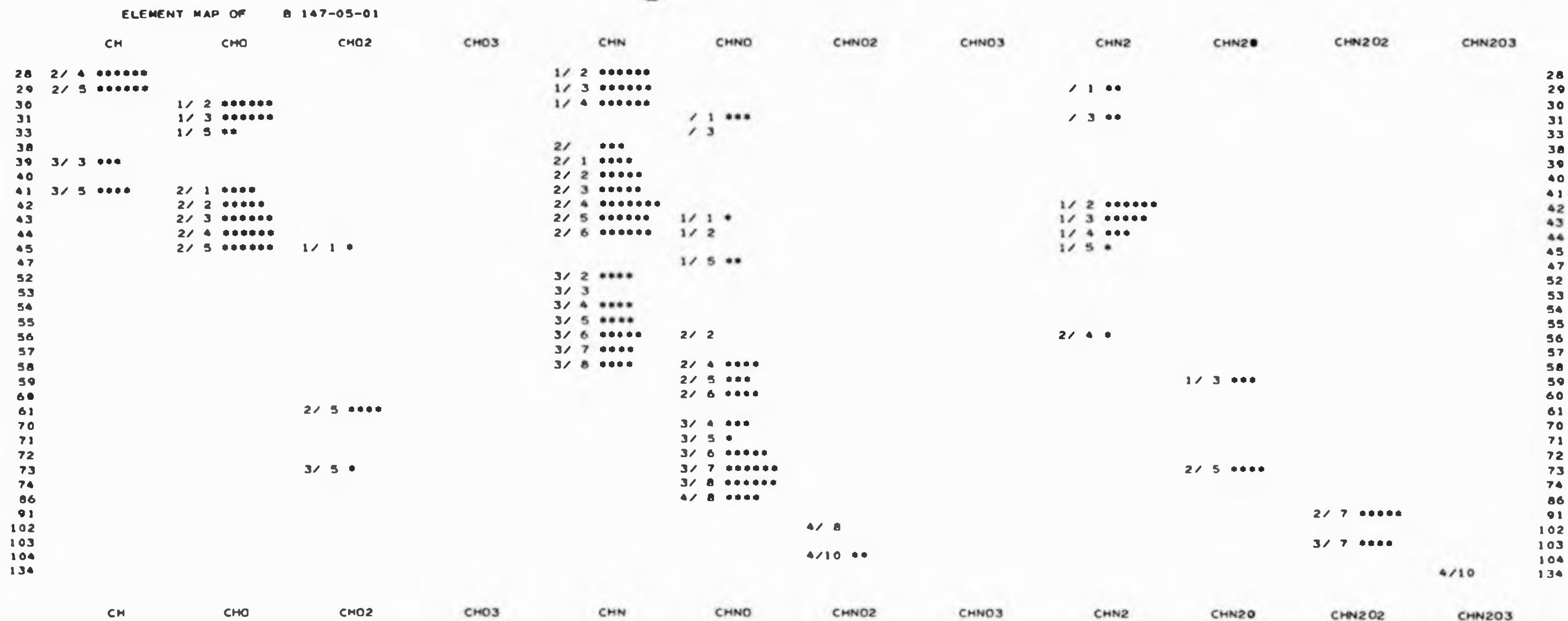

Fig. 2. High resolution mass spectrum in the form of an element map (Biemann *et al.* 1964) of NDEIA, elemental composition $C_4H_{10}N_2O_3$.

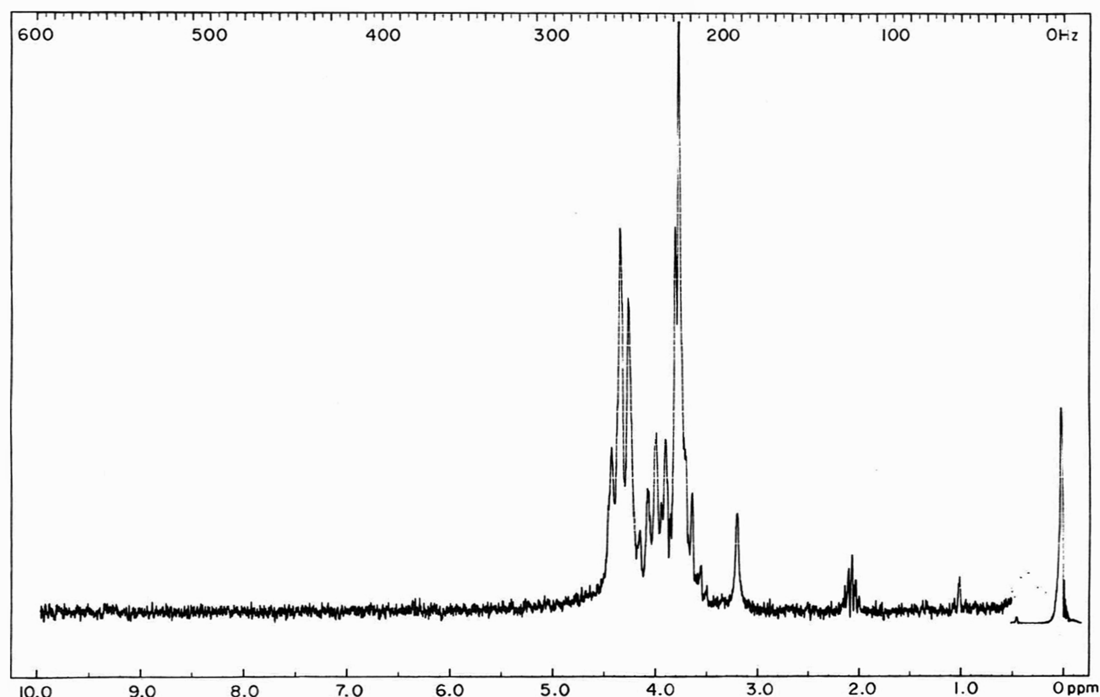

Fig. 3. Proton magnetic resonance spectrum of NDEIA.

(50 nm) column (Waters Associates) using tetrahydrofuran at 1·5 ml/min as a carrier, and the material eluting at the retention time of NDEIA was collected and re-concentrated. Final clean-up of the extract was carried out on a μ Bondapak-CN column using hexane–methylene chloride–methanol, 85:10:5 by vol., at 2 ml/min. The material eluting at the retention time of NDEIA was concentrated to 50 μl under vacuum, and transferred to a pyrex-glass capillary tube (1 mm ID). The solvent was allowed to evaporate at reduced pressure and the tube was sealed with a small flame. The sealed glass capillary tube was used to store the sample prior to mass-spectrometric analysis, at which time the capillary tube was cut to a length of 15 mm and inserted into the instrument *via* the direct sample introduction system.

RESULTS

Figure 4a is the TEA–HPLC chromatogram of 25 μl of the isolate from shampoo sample S1 containing 280 ng NDEIA/g. The concentrate has a relatively large initial solvent front doublet, followed by two small peaks prior to the peak eluting at the retention time of NDEIA. Figure 4b is the chromatogram from shampoo sample S7, containing more than 70 ng NDEIA/g. The peak at the retention time of NDEIA is clearly discernible, with a signal-to-noise ratio better than 4:1. The peaks prior to NDEIA in the case of sample S1 are more pronounced than the NDEIA peak. No attempt was made to characterize the materials eluting in the solvent front or in the peaks prior to NDEIA.

The NDEIA concentrations measured in ng/g (ppb) in cosmetics, lotions and hair shampoos are shown in Table 1. A duplicate determination of the NDEIA concentration in each product was carried out in the presence of 1% triethanolamine plus 0·1% sodium nitrite. In no case did added triethanolamine and nitrite significantly enhance the reported

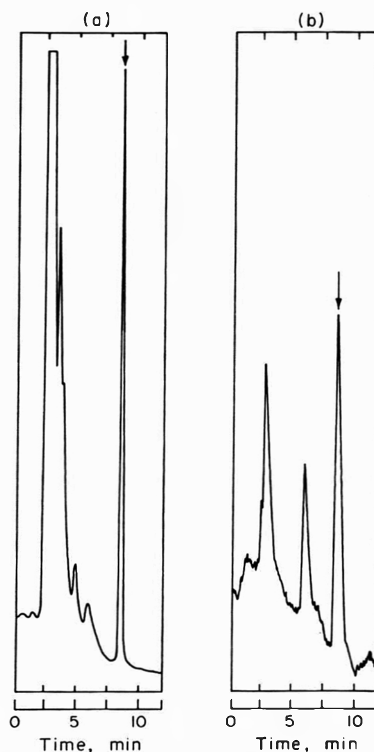

Fig. 4. TEA–HPLC chromatograms for 25 μl of concentrates on μ Porasil column, with 2 ml/min acetone–isooctane, 40:60 v/v; (a) from shampoo sample S1, TEA attenuation × 16; (b) from shampoo sample S7, TEA attenuation × 4 (NDEIA peaks arrowed).

NDElA concentration. In the right-hand column of Table 1 the reported NDElA content has been corrected for recovery efficiency.

The material with the retention time of NDElA in the HPLC peak of cosmetic sample C4 was isolated and found by high-resolution mass spectrometry to show the following fragments characteristic of NDElA: *m/e* 104·0717 (calculated for $C_4H_{10}NO_2$: 104·0712), *m/e* 103·0510 (calculated for $C_3H_7N_2O_2$, 103·0508) and *m/e* 91·0515 (calculated for $C_2H_7N_2O_2$, 91·0508). Figure 5 shows the key fragments produced from NDElA in the mass spectrometer, and the exact masses of these fragments used in the identification of the unknown material from cosmetic sample C4.

DISCUSSION

Because triethanolamine is an effective wetting agent and emulsifier, conventional solvent–solvent extraction techniques could not be used to extract NDElA from a mixture containing triethanolamine. To ensure high recoveries, NDElA was isolated, therefore, on a silica-gel column and then eluted with acetone. It was demonstrated that any nitrite present in the sample would readily nitrosate ethanolamine, even during stirring or mild heating. For this reason, ammonium sulphamate was added at the beginning of the analysis procedure to prevent NDElA formation during analysis. To prevent further NDElA formation during analysis, the sample was not subjected to temperatures in excess of those of the surroundings. Additional proof that NDElA was not formed during analysis was the fact that identical quantitative NDElA results were obtained both with and without the addition of 1% triethanolamine plus 0·1% sodium nitrite to each sample.

The TEA was used as the detector for the HPLC (Fine, Rounbehler, Silvergleid & Ross, 1977c). The selectivity of the TEA–HPLC for nitroso compounds has been demonstrated in complex systems such as cooked foodstuffs (Fine, Ross, Rounbehler, Silvergleid & Song, 1976), herbicides (Fine *et al.* 1977b) and cutting fluids (Fan *et al.* 1977). There is no positive response on the TEA either from di- and/or triethanolamine or from a solution containing diethanolamine, triethanolamine and nitrite; nor is the response to NDElA enhanced by the presence of triethanol-

Table 1. N-*Nitrosodiethanolamine content of cosmetics, lotions and shampoos*

Sample		Recovery efficiency (%)	NDElA content (ng/g)		
			Sample	Sample + $NaNO_2$ + $(C_2H_4OH)_3N$	Corrected*
Cosmetic	C1	103	125	80	100
	C2	—	41	42	>40
	C4	—	27,000	23,000	25,000†
	C4	96	47,000	48,000	49,000
	C5	41	1800	1200	3700
	C6	14	1200	890	>1200
	C7	—	trace	—	trace
Lotion	L1	49	48	51	100
	L1	50	5–10	—	trace
	L2	24	trace	—	trace
	L3	23	14	24	83
	L5	52	ND	—	ND
	L6	—	trace	—	trace
	L7	—	trace	—	trace
	L8	—	5–10	—	trace
	L9	—	trace‡	trace‡	trace
	L10	—	trace	—	trace
	L11	7	140	94	>140
	L13	36	14	20	47
	L14	10	22	12	>22
Shampoo	S1	103	280	250	260
	S3	—	5–10	—	trace
	S4	43	46	44	100
	S5	—	trace‡	trace‡	trace
	S6	—	ND	—	ND
	S7	19	70	40	>70
	S8	—	5–10	—	trace
	S9	30	18	23	68
	S10	11	17	27	>27

NDElA = *N*-nitrosodiethanolamine ND = Not detected (less than 1 ng/g) trace = <10 ng/g.
* This figure was obtained by correcting for recovery efficiency the average NDElA content of the sample and sample + $NaNO_2$ + $(C_2H_4OH)_3N$.
† Identity of NDElA confirmed by high-resolution mass spectrometry.
‡ Quadruplicate analyses.

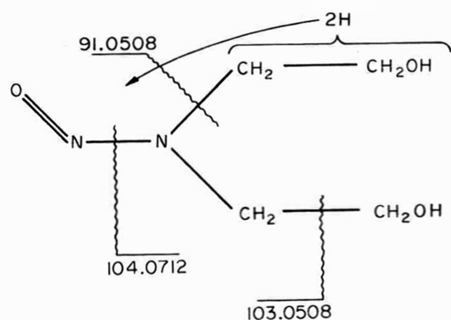

Fig. 5. Diagrammatic representation of characteristic mass-spectral fragmentation pathways of NDEIA.

amine, diethanolamine, nitrite or all three. The TEA is selective for compounds with a labile nitrosyl group, such as *N*-nitroso compounds, organic nitrites and some organic nitrates (Fine, Rufeh & Lieb, 1974; Fine, Rufeh, Lieb & Rounbehler, 1975).

The recovery efficiency for different formulations varied from a high of 103% to a low of 7% (see Table 1). We attribute the wide variability in recovery efficiency to the fact that no single analytical procedure can work well for products which vary from a solid soap, to a thick cream or hair shampoo. Furthermore, the ingredients of each formulation differ widely, and different ingredients would be expected to alter radically the recovery efficiency from both the initial ethyl acetate partition and the subsequent elution from the silica-gel column. Because low recoveries may bias the data, those formulations with recovery efficiencies of less than 20% were not corrected in Table 1, but were instead reported as greater than the highest measured value.

To ensure that the compound eluting on μ Porasil at the retention time of NDEIA was due only to NDEIA, the concentrate remaining after the quantitative TEA–HPLC analysis had been completed on sample C4 (see Table 1), was also quantitatively chromatographed as a single peak eluting at the retention time of NDEIA on a Styragel 50-nm column and on a μ Bondapak-CN column. After isolation and purification on these three columns the concentrate was collected for high-resolution mass spectrometry. Figure 6 (bottom) shows the TEA–HPLC chromatograms on the three different columns for the concen-

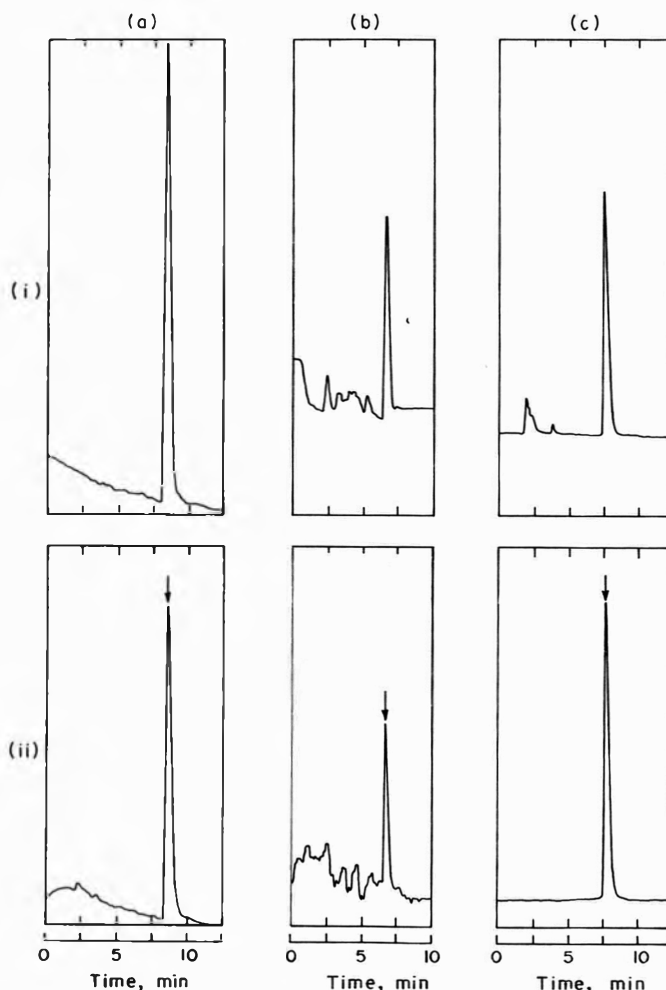

Fig. 6. TEA–HPLC chromatograms for (i) authentic NDEIA and (ii) isolate from cosmetic sample C4 (NDEIA peaks arrowed): (a) on μ Bondapak-CN with hexane–methylene chloride–methanol, 85:10:5 by vol. at 2 ml/min; (b) on μ Styragel 50 nm with tetrahydrofuran at 1·5 ml/min; (c) on μ Porasil with hexane–acetone, 60:40 v/v at 2 ml/min.

trate from cosmetic sample C4. Finally, the identity of the peak eluting at the retention time of NDElA was independently verified as NDElA by high resolution mass spectrometry.

High-resolution mass spectrometry rather than low-resolution mass spectrometry was chosen for the characterization of the concentrate from cosmetic sample C4 because the former affords exact masses which may then be assigned elemental compositions, while the latter merely affords nominal masses. The distinction between the two can be made more clearly by referring to *m/e* 91 in the low-resolution mass spectrum of NDElA (Fig. 1). The presence of an abundant *m/e* 91 in the mass spectra of organic compounds is most frequently due to the tropylium ion $C_7H_7^+$ with an exact mass of 91·0548. However such is not the case for NDElA, for which the exact mass of *m/e* 91 was measured as 91·0501, which corresponds to the elemental composition $C_2H_7N_2O_2$ (91·0508) and is listed in the element map (Fig. 2) in the N_2O_2 column.

Only three peaks in the high-resolution mass spectrum of NDElA (Fig. 2) are necessary to characterize the compound fully, and these are shown in Fig. 5. The loss of NO from the molecular ion gives rise to $(HOCH_2{-}CH_2)_2N^+$, a weak but highly characteristic peak at *m/e* 104·0712. Fragmentation of the C–C bond with charge retention on the carbon atom bearing a nitrogen leads to the fragment $HOCH_2{-}CH_2{-}N(NO)^+CH_2$ at *m/e* 103·0508. Finally cleavage of the C–N bond with transfer of two hydrogens affords the protonated N-nitrosomonoethanolamine $HOCH_2{-}CH_2{}^+NH_2(NO)$ at *m/e* 91·0508.

As indicated in Fig. 3, other TEA-responsive compounds were present in most of the products that were tested (often at mole concentrations considerably in excess of those reported for NDElA). Future work is planned to isolate these TEA-responsive compounds so that they can be identified by means of conventional chemical techniques.

It is instructive to assess possible human exposure to NDElA from these products. Shampoo S1 is typically used in 10–15 g quantities, giving an NDElA exposure for each use of about 3 μg. Lotion L1 is used in approximately 10–20 g quantities, giving a daily exposure of about 2 μg NDElA. This product is also recommended for infant use at each napkin change, which would lead to a considerably greater exposure relative to body weight. Cosmetic C4 is used in approximately 1–2 g quantities, giving a daily exposure to 50–100 μg NDElA. Individuals such as actresses and models may be exposed to even higher levels.

It is not possible at the present time to assess properly the potential hazard to man from NDElA since the carcinogencity of NDElA has been studied only in the case of ingestion and not when absorbed through the skin. However, since triethanolamine is a wetting agent, a significant amount of NDElA applied to the skin may be absorbed. NDElA is a known liver carcinogen in rats and belongs to a group of compounds demonstrated to be carcinogenic to all species so far tested. The data in Table 1 indicate a wide variation in NDElA concentration for similar products. It is likely, therefore, that the technology for eliminating the carcinogenic impurity would be available should the mechanism of contamination be identified.

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REFERENCES

Biemann, K., Bommer, P. & Desiderio, D. M. (1964). Element-mapping, a new approach to the interpretation of high resolution mass spectra. *Tetrahedron Lett.* no. 26, p. 1725.

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.

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

Fan, T. Y., Morrison, J., Rounbehler, D. P., Ross, R., Fine, D. H., Miles, W. & Sen, N. P. (1977). N-Nitrosodiethanolamine in synthetic cutting fluids: a part-per-hundred impurity. *Science, N.Y.* **196,** 70.

Fine, D. H., Ross, R., Rounbehler, D. P., Silvergleid, A. & Song, L. (1976). Analysis of nonionic nonvolatile N-nitroso compounds in foodstuffs. *J. agric. Fd Chem.* **24,** 1069.

Fine, D. H., Rounbehler, D. P., Belcher, N. M. & Epstein, S. S. (1976). *N*-Nitroso compounds: Detection in ambient air. *Science, N.Y.* **192,** 1238.

Fine, D. H., Rounbehler, D. P., Fan, T. Y. & Ross, R. (1977a). Human exposure to N-nitroso compounds in the environment. In *Cold Spring Harbor Conferences on Cell Proliferation, Origins of Human Cancer*, Vol. 4. Cold Spring Harbor Laboratory. In press.

Fine, D. H., Rounbehler, D. P., Pellizzari, E. D., Bunch, J. E., Berkley, R. W., McCrae, J., Bursey, J. T., Sawicki, E., Krost, K. & DeMarrais, G. (1976). N-Nitrosodimethylamine in air. *Bull. env. contam. & Toxicol. (U.S.)* **15,** 739.

Fine, D. H., Rounbehler, D. P., Rounbehler, A. & Silvergleid, A. (1977b). Determination of dimethylnitrosamine in air, water and soil by thermal energy analysis measurements in Baltimore, Maryland. *Envir. Sci. and Technol.* **11,** 581.

Fine, D. H., Rounbehler, D. P., Silvergleid, A. & Ross, R. (1977c). Trace analysis of polar and nonpolar N-nitroso compounds by combined high performance liquid chromatography and thermal energy analysis. In *Proc. Second Intl. Symposium on Nitrite in Meat Products* (Oct. 1976). Edited by B. J. Timbergen and B. Krol. p. 191. Center for Agricultural Publishing and Documentation, Wageningen, Netherlands.

Fine, D. H., Rufeh, F. & Lieb, D. (1974). Group analysis of volatile and non-volatile N-nitroso compounds. *Nature, Lond.* **247,** 309.

Fine, D. H., Rufeh, F., Lieb, D. & Rounbehler, D. P. (1975). Description of the Thermal Energy Analyzer (TEA) for trace determination of volatile and nonvolatile *N*-nitroso compounds. *Analyt. Chem.* **47,** 1188.

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

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

Magee, P. N. & Barnes, J. M. (1956). The production of malignant primary hepatic tumours in the rat by feeding dimethylnitrosamine. *Br. J. Cancer* **10**, 114.

Magee, P. N., Montesan, R. & Preussmann, R. (1976). N-Nitroso compounds and related carcinogens. In *Chemical Carcinogens*. ACS Monograph no. 173. Edited by C. E. Searle, p. 491. American Chemical Society.

Merck Index (1976). *An Encyclopedia of Chemicals and Drugs*. 9th ed., p. 1072. Merck & Co., Inc., Rahway, New Jersey.

Scanlan, R. A. (1975). N-Nitrosamines in foods. *Crit. Rev. Fd Technol.* **5**, 357.

Scheltz, I., Abidi, S. & Hoffman, D. (1977). Tumorigenic agents in unburned processed tobacco: N-nitrosodiethanolamine and 1,1-dimethylhydrazine. *Cancer Lett.* **2**, 125.

Sen, N. P., Seaman, S. & Miles, W. F. (1976). Dimethylnitrosamine and nitrosopyrrolidine in fumes produced during the frying of bacon. *Fd Cosmet. Toxicol.* **14**, 167.

Fd Cosmet. Toxicol. Vol. 15, pp. 431–435. Pergamon Press 1977. Printed in Great Britain

EFFECT OF L-TRYPTOPHAN ON DIETHYLNITROSAMINE AND 3′-METHYL-4-*N*-DIMETHYLAMINOAZOBENZENE HEPATOCARCINOGENESIS

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Abstract—The effect of L-tryptophan on hepatocarcinogenesis induced by diethylnitrosamine (DENA) and 3′-methyl-4-*N*-dimethylaminoazobenzene (3′-MeDAB) was studied in Wistar male rats. Tryptophan decreased the incidence of liver tumours in both DENA- and 3′-MeDAB-fed animals but had no effect on the location and incidence of oesophageal tumours among DENA-fed animals. The incidence of anaplastic liver carcinomas was higher among the animals given diet without tryptophan supplement than among those given the tryptophan supplement.

INTRODUCTION

Tryptophan and its metabolites have been the object of intensive research since Dunning, Curtis & Maun (1950) found that there was a higher incidence of bladder tumours in rats fed 2-acetylaminofluorene together with tryptophan than in rats fed 2-acetylaminofluorene alone. *o*-Aminophenol metabolites of tryptophan were also shown to produce bladder tumours when cholesterol pellets containing these metabolites were inserted into the mouse bladder (Allen, Boyland, Dukes, Horning, & Watson, 1957). Besides bladder tumours, tryptophan was shown by Kawachi, Hirata & Sugimura (1968) to increase the incidence of liver tumours induced by diethylnitrosamine (DENA). The liver is known to be the main site of metabolism for the tryptophan–nicotinic acid pathway, because the key enzyme, tryptophan oxygenase, is present only in this organ.

The purpose of the present experiment was to extend knowledge of the role of L-tryptophan in liver carcinogenesis by studying its effect on tumour development in rats receiving a different carcinogen, 3′-methyl-4-*N*-dimethylaminoazobenzene (3′-MeDAB). This agent was selected because it had been shown that a synthetic derivative of *N*-methyl-4-aminoazobenzene, *N*-benzoyloxy-*N*-methyl-4-aminoazobenzene, reacted *in vitro* at pH 7 with cell macromolecules and with some amino acids, one of which was tryptophan (Poirier, Miller, Miller & Sato, 1967). Although the mechanism of action of these carcinogens is still undetermined, carcinogenic alkylating agents like DENA and azo dyes are known to react with cellular macromolecules and these reactions may provide the basis for the carcinogenicity of such compounds.

EXPERIMENTAL

L-Tryptophan (50 g, from ICN Pharmaceuticals, Inc., Cleveland, Ohio) was thoroughly mixed with 400 g Wayne laboratory meal using a mortar and pestle. This mixture was then blended with a larger portion of meal to yield a final weight of 5 kg (1% tryptophan). Diets containing 0·05% 3′-MeDAB (Eastman Kodak Co., Rochester, N.Y.) were prepared similarly by first mixing 2·5 g of carcinogen with 400 g of basal diet. Diets were prepared twice weekly and stored in a cold room until fed. DENA (Eastman Kodak Co.) was given in the drinking-water as a 0·002% (w/v) solution.

Young male Wistar rats (Microbiological Associates, Bethesda, Md) were fed Wayne laboratory meal up to the age of 5 wk, when they were divided into six groups of matched weights (121·2 ± 1·5 g). Basal diet was given to groups I and III and 1% tryptophan diet to groups II and IV. DENA was administered to groups III and IV in the drinking-water (Table 1). Group V was given 0·05% 3′-MeDAB in the basal diet and group VI 0·05% 3′-MeDAB in the 1% tryptophan diet (see Table 2). Rats were kept on DENA for 128 days and on 3′-MeDAB for 96 days. Thereafter basal or 1% tryptophan diet was given as before until day 166, when the rats were killed.

Animals were weighed weekly for 2 months and then every 2 wk for the rest of the experiment. Tissues were fixed in 10% buffered neutral formalin; routine techniques were used to prepare histological slides. Histopathological criteria for the classification of liver tumours were based on those used by Reuber & Lee (1968).

RESULTS

Figure 1 shows the growth curves obtained for each group. There was no difference in weights between the control group and the group receiving supplementary tryptophan. The weights of animals fed DENA with tryptophan supplement were somewhat higher than those of animals fed DENA only up to the age of 20 wk. Thereafter the weights of both of these groups went down, possibly owing to the chronic bronchopneumonia which seemed to be more severe in rats in the DENA experiment; the weight decrease began sooner in the group receiving DENA alone. The groups given 3′-MeDAB showed considerably

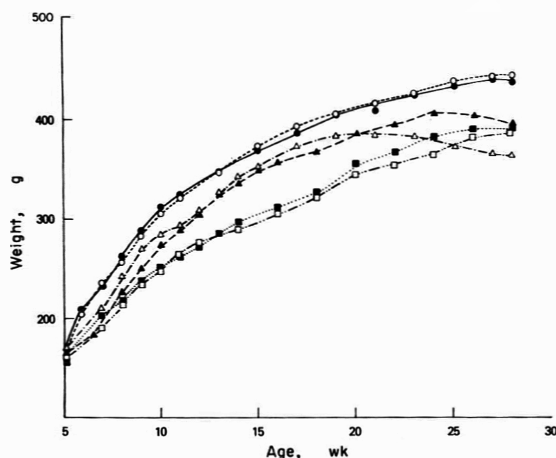

Fig. 1. Mean body weights of groups of 20–30 rats given the following treatments: control (●——●), 1% tryptophan (○——○), DENA (▲-----▲), DENA + 1% tryptophan (△-·-·-·-△), 3′-MeDAB (■····■), 3′-MeDAB + 1% tryptophan (□-··-··-··-□).

less weight-gain than the control group. Up to the age of 19 wk, the weight gains of the groups fed carcinogen with and without tryptophan were the same, but thereafter the rats on carcinogen without tryptophan supplement gained somewhat faster owing to the rapid growth of the liver tumours.

Food intakes of groups V and VI, measured when the animals had been on the diet for 4–5 wk, were 14·9 and 16·7 g/rat/day in wk 4 and 21·1 and 22·4 g/rat/day, respectively, in wk 5 of the experiment.

The drinking solution of DENA for groups III and IV was prepared each week and the amount given was recorded. The amount of solution taken by each animal daily was calculated periodically (Table 1); there was no significant difference between the groups with and without tryptophan.

As shown in Table 2, tryptophan decreased the incidence of liver tumours in both DENA- and 3′-MeDAB-fed animals. Tryptophan slightly increased the liver weights of group II as compared with group I. When liver weights were calculated per tumour-bearing animal, groups without tryptophan had higher liver weights because of the added weight of the liver tumours. However, the difference was only significant among 3′-MeDAB fed animals. The incidence of anaplastic carcinomas was higher among the animals in groups III and V than among tryptophan-supplemented animals. The tryptophan supplement led to trabecular or adenomatous well-differentiated tumours. There were five haemangioendothelial sarcomas in DENA-fed animals without tryptophan and only one in the tryptophan-supplemented group. Tryptophan feeding seemed to promote cirrhotic changes in the liver of 3′-MeDAB fed animals, whereas the livers of the animals on the carcinogen alone had large necrotic areas which persisted without obvious evidence of regeneration or fibrosis. Around the borders of these necrotic areas were basophilic hyperplastic cells, which in later stages progressed to poorly differentiated hepatocellular carcinomas. Proliferation of bile ducts was common among 3′-MeDAB-fed animals, whereas it was absent among rats fed DENA.

There was no difference in the location and incidence of oesophageal tumours among animals fed DENA with and without tryptophan. Both carcinogens caused ulceration of the glandular stomach and the small intestine; tryptophan feeding did not have any effect on the histological picture of these organs.

DISCUSSION

The effect of nutritional and dietary factors on azo-dye carcinogenesis has been well established (Day, Payne & Dinning, 1950; Elson, 1958; Kensler, Sugiura, Young, Halter & Rhoads, 1941; Miller, Miner, Rusch & Baumann, 1941). There is, however, much less information about the effect of dietary factors on nitrosamine carcinogenesis. A great deal about the metabolism of both of these groups of carcinogens has been learned, but the mechanism of their carcinogenic action is still unclear. Both nitrosamines and azo compounds are converted to the ultimate carcinogens by microsomal drug-metabolizing enzymes. Any dietary or other factors that decrease their carcinogenicity might do so at different levels of cellular metabolism. Such factors might affect the balance between activating and inactivating reactions of drug metabolism, or inhibit the reactions between the chemically reactive carcinogenic metabolites and cellular macromolecules (e.g. proteins, DNA and RNA) or possibly improve the repair mechanisms which come into play after carcinogen-produced damage.

This study did not confirm previous reports of enhancement of DENA carcinogenesis by L-tryptophan (Kawachi *et al.* 1968). One difference between the earlier study and the present one was a shorter period of carcinogen feeding. This was necessary because animals given DENA without tryptophan became anaemic and weak due to bleeding into the abdominal cavity. The tumour incidence in this group was 88% compared with 17% in the work reported earlier (Kawachi *et al.* 1968). In the present study the protective effect of tryptophan feeding was more evident for 3′-MeDAB than for DENA. It is possible that an even shorter period of DENA feeding than that used in the present study might have shown the effect of the dietary factor more clearly.

Pyridoxine is an important cofactor in tryptophan metabolism, and changes in the metabolism of this

Table 1. *Water intake by Groups III (DENA) and IV (DENA + tryptophan)*

Wk no.	Water intake (ml/rat/day) by Group III	Group IV
3	14	14
8	20	19
12	17	18
17	23	22

DENA = Diethylnitrosamine

Table 2. *Effect of L-tryptophan on hepatic carcinogenesis by diethylnitrosamine and 3'-methyl-4-N-dimethylaminoazobenzene; pathological changes*

Group	No. of rats/group	Treatment	No. of rats with hepatic tumours	Liver weight/100 g body weight†	Hepatocellular tumours		Haemangio-endothelial sarcoma	Cholangioma	Metastasis
					Anaplastic	Well-differentiated‡			
I	20	None	0	3·1 ± 0·1	—	—	—	—	—
II	20	Trp	0	3·5 ± 0·1*	—	—	—	—	—
III	25	DENA	22 (88%)	6·4 ± 0·9§	13 (59%)	4 (18%)	5 (23%)	—	5 (23%)
IV	29	DENA + Trp	17 (59%)*	5·0 ± 0·5§	8 (47%)	8 (47%)	1 (6%)	—	6 (33%)
V	29	3'-MeDAB	24 (83%)	14·3 ± 1·4§**	21 (88%)	3 (13%)	—	—	10 (42%)
VI	30	3'-MeDAB + Trp	12 (40%)***	9·2 ± 1·9§	5 (42%)	5 (42%)	—	2 (16%)	4 (33%)

Trp = L-Tryptophan DENA = Diethylnitrosamine 3'-MeDAB = 3'-Methyl-4-*N*-dimethylaminobenzene

†Values are means ± SEM.
‡Trabecular or adenomatous.
§Per tumour-bearing animal.
Values marked with asterisks differ significantly from the corresponding value with or without tryptophan: *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

amino acid induced by aromatic amines have been shown to be reversed by an increase in the amount of dietary pyridoxine (Dyer & Morris, 1961). Because of the role of pyridoxine in tryptophan metabolism it seemed possible that the difference between the outcome of this study and the study by Kawachi *et al.* (1968) might have resulted from differences in dietary pyridoxine in the two studies. Because of this possibility, samples of commercial laboratory meal for rats were obtained from Japan. Analyses for pyridoxine, however, showed no differences between the Japanese diet and the one used in the present study.

Little is known about the effect of azo compounds and nitrosamines on trytophan metabolism. The observed protective effect of tryptophan against liver carcinogenesis agrees with the finding by Okajima, Hiramatsu, Motomiya, Iriya, Ijuin & Ito (1971) that tryptophan completely prevented liver tumours induced by dibutylnitrosamine in rats. Tryptophan has also been shown to have a protective effect against cholangiofibrosis and cholangiosarcoma of hamster liver induced by 2-fluorenylacetamide (Oyasu, Katajima, Hopp & Sumie, 1972).

An early biochemical lesion in liver carcinogenesis is inhibition of protein synthesis (Magee, 1958). Electron-microscopic and biochemical studies with DENA and 3'-MeDAB have revealed detachment of ribosomes from the membrane of the endoplastic reticulum, loss of the aggregate structure characteristic of polysomes, and changes in mRNA profile (Emmelot & Benedetti, 1960; Ketterer, Holt & Ross-Mansell, 1967; Molbert, Hill & Buchner, 1962; Parish & Kirkby, 1966; Porter & Bruni, 1959). Inhibition of protein synthesis parallels the breakdown of polysomal aggregates (Villa-Trevino, 1967). On the other hand, tryptophan has an opposite effect on the biochemistry and morphology of the liver; it increases protein synthesis (Fiegelson, Fiegelson & Fancher, 1959; Sidransky, Sarma, Bongiorno & Verney, 1968), polyribosomal mRNA (Murty & Sidransky, 1972; Oravec & Korner, 1971) and polyadenylic acid-containing mRNA in the cytoplasm (Murty, Verney & Sidransky, 1976) and causes a shift in hepatic polyribosomes toward denser aggregates (Park, Henderson & Swan, 1973; Sidranksy *et al.* 1968). If carcinogens damage microsomal macromolecules, and if on the other hand tryptophan affects the repair mechanism of ribosomes by enhancing the maturation of mRNA and its nucleocytoplasmic transport (Murty *et al.* 1976), this could explain the protective effect of tryptophan against liver carcinogenesis. Methionine, another amino acid with a protective effect on amino azo-dye carcinogenesis (Day *et al.* 1950; Griffin, Clayton & Baumann, 1949), decreases the proportion of the liver ribosomes found in monomer and dimer fractions and increases the proportion in polysomes (Park *et al.* 1973). It has been suggested that the role of carcinogens is to increase tryptophan metabolism in the liver, possibly by increasing the activity of tryptophan oxygenase (Dyer & Morris, 1961). This would explain an increased need for this amino acid for normal liver function. Animals fed 3'-MeDAB without tryptophan supplement had large areas of liver necrosis. In animals given the tryptophan supplement, necrosis led to a prominent cirrhotic reaction, which may be taken as a morphological sign of increased 'repair' initiated by tryptophan.

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REFERENCES

Allen, M. J., Boyland, E., Dukes, C. E., Horning, E. S. & Watson, J. G. (1957). Cancer of urinary bladder induced in mice with metabolites of aromatic amines and tryptophan. *Br. J. Cancer* **11**, 212.

Day, P. L., Payne, L. D. & Dinning, J. S. (1950). Procarcinogenic effect of vitamin B_{12} on *p*-dimethylaminoazobenzene-fed rats. *Proc. Soc. exp. Biol. Med.* **74**, 854.

Dunning, W. F., Curtis, M. R. & Maun, M. E. (1950). The effect of added dietary tryptophan on the occurrence of 2-acetylaminofluorene-induced liver and bladder cancer in rats. *Cancer Res.* **10**, 454.

Dyer, H. M. & Morris, H. P. (1961). An effect of N-2-fluorenylacetamide on the metabolism of tryptophan in rats. *J. natn. Cancer Inst.* **26**, 315.

Elson, L. A. (1958). Some dynamic aspects of chemical carcinogenesis. *Br. med. Bull.* **14**, 161.

Emmelot, P. & Benedetti, E. L. (1960). Changes in the fine structure of the rat liver cells brought about by dimethylnitrosamine. *J. biophys. biochem. Cytol.* **7**, 393.

Fiegelson, P., Fiegelson, M. & Fancher, C. (1959). Kinetics of liver nucleic acid turnovers during enzyme induction in the rat. *Biochim. biophys. Acta* **32**, 133.

Griffin, A. C., Clayton, C. C. & Baumann, C. A. (1949). The effects of casein and methionine on the retention of hepatic riboflavin and on the development of liver tumors in rats fed certain azo dyes. *Cancer Res.* **9**, 82.

Kawachi, T., Hirata, Y. & Sugimura, T. (1968). Enhancement of N-nitrosodiethylamine hepatocarcinogenesis by L-tryptophan in rats. *Gann* **59**, 523.

Kensler, C. J., Sugiura, K., Young, N. F., Halter, C. R. & Rhoads, C. P. (1941). Partial protection of rats by riboflavin with casein against liver cancer caused by dimethyl-aminoazobenzene. *Science, N.Y.* **93**, 308.

Ketterer, B., Holt, S. J. & Ross-Mansell, P. (1967). The effect of a single intraperitoneal dose of the hepatocarcinogen 4-dimethylaminoazobenzene on the rough-surfaced endoplasmic reticulum of the liver of the rat. *Biochem. J.* **103**, 692.

Magee, P. N. (1958). Inhibition of protein synthesis in rat liver by dimethylnitrosamine *in vivo*. *Biochem. J.* **70**, 606.

Miller, J. A., Miner, D. L., Rusch, H. P. & Baumann, C. A. (1941). Diet and hepatic tumor formation. *Cancer Res.* **1**, 699.

Molbert, E., Hill, K. u. Buchner, F. (1962). Die Kanzerisierung der Leber-parenchymzelle durch Diaethylnitrosamin im elektronmikroskopischen Bild. *Beitr. Path. Anat.* **126**, 218.

Murty, C. N. & Sidransky, H. (1972). The effect of tryptophan on messenger RNA of the livers of fasted mice. *Biochim. biophys. Acta* **262**, 328.

Murty, C. N., Verney, E. & Sidransky, H. (1976). Effect of tryptophan on polyriboadenylic acid and polyadenylic acid-messenger ribonucleic acid in rat liver. *Lab. Invest.* **34**, 77.

Okajima, E., Hiramatsu, T., Motomiya, Y., Iriya, K., Ijuin, M. & Ito, N. (1971). Effect of dl-tryptophan on tumori-

genesis in the urinary bladder and liver of rats treated nitrosodibutylamine. *Gann* **62,** 163.

Oravec, M. & Korner, A. (1971). Stimulation of ribosomal and DNA-like RNA synthesis by tryptophan. *Biochim. biophys. Acta* **247,** 404.

Oyasu, R., Kitajima, T., Hopp, M. L. & Sumie, H. (1972). Enhancement of urinary bladder tumorigenesis in hamsters by coadministration of 2-acetylaminofluorene and indole. *Cancer Res.* **32,** 2027.

Parish, J. H. & Kirkby, K. S. (1966). Patterns of mRNA synthesis during rat liver carcinogenesis induced by diethylnitrosamine and thioacetamide. *Biochim. biophys. Acta* **114,** 198.

Park, O. J., Henderson, L. M. & Swan, P. B. (1973). Effect of the administration of single amino acids on ribosome aggregation in rat liver. *Proc. Soc. exp. Biol. Med.* **142,** 1023.

Poirier, L. A., Miller, J. A. Miller, E. C. & Sato, K. (1967). N-Benzoyloxy-N-methyl-4-aminoazobenzene: Its carcinogenic activity in the rat and its reactions with proteins and nucleic acids and their constitutents *in vitro. Cancer Res.* **27,** 1600.

Porter, K. R. & Bruni, C. (1959). An electron microscope study of the early effects of 3-Me-DAB on rat liver cells. *Cancer Res.* **19,** 997.

Reuber, M. D. & Lee, C. W. (1968). Effect of age and sex on hepatic lesions in Buffalo strain rats ingesting diethylnitrosamine. *J. natn. Cancer Inst.* **41,** 1133.

Sidransky, H., Sarma, D. S. R., Bongiorno, M. & Verney, E. (1968). Effect of dietary tryptophan on hepatic polyribosomes and protein synthesis in fasted mice. *J. biol. Chem.* **243,** 1123.

Villa-Treviño, A. (1967). A possible mechanism of inhibition of protein synthesis by dimethylnitrosamine. *Biochem. J.* **105,** 625.

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VOLATILE NITROSAMINES FROM ION-EXCHANGE RESINS

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Abstract—Deionized water was analysed for volatile nitrosamines, and was found to contain minute amounts of these compounds. Ion-exchange resins, including those used in water deionization plants, were examined and the anion exchangers were identified as the source of the nitrosamines. Gas chromatography combined with chemiluminescence and mass spectrometry was used to identify and quantify the nitrosamines. The relevance of these observations to food analysis is discussed.

INTRODUCTION

Nitrosamines have been detected in cured meats and particularly in fried bacon, in which dimethylnitrosamine (DMNA) and *N*-nitrosopyrrolidine (NPYR) are frequently found (Crosby, Foreman, Palframan & Sawyer, 1972; Fazio, White, Dusold & Howard, 1973; Gough, Goodhead & Walters, 1976; Pensabene, Fiddler, Gates, Fagan & Wasserman, 1974). DMNA has also been detected occasionally in cheese and fish (Crosby *et al.* 1972; Fazio, Damico, Howard, 1973; Gough, Goodhead & Walters, 1976; Pensabene, Fiddler, Gates, Fagan & Wasserman, only rarely. In a recent study at this Laboratory (Gough, McPhail, Webb, Wood & Coleman, 1977), no volatile nitrosamines were detected in samples of nearly 200 varieties of foods typical of the United Kingdom diet. The detection limit for each nitrosamine was 1 μg/kg of food, and the measurements were based on combined gas chromatography and mass spectrometry.

The recent introduction of a more sensitive detector for nitrosamines has enabled the detection limit to be reduced to 0·01 μg/kg of food, and these food extracts have been re-examined. The detector is based on the chemiluminescent emission resulting from interaction of ozone and nitric oxide. The nitric oxide is generated catalytically from the nitrosamine eluted from the chromatograph (Fine, Rufeh, Lieb & Rounbehler, 1975). Dialkyl nitrosamines, usually DMNA and methylethylnitrosamine, were detected in 92% of the samples. Such widespread occurrence, particularly of the asymmetric nitrosamine, which has not been reported to occur even in cured meat, indicated an artefact arising either from a non-specific response of the detector or from inadvertent introduction or generation of these nitrosamines during sample preparation. Foodstuffs are normally slurried in deionized water and the resulting mixture is steam-distilled. Steam is also generated from deionized water, so that if any nitrosamines were present in this water supply they would be distilled with those present in the food, and thus contribute to the apparent nitrosamine content of the food.

In the work reported here, we analysed samples of the water used for these preparations and found minute amounts of DMNA in some cases. Water was also examined after overnight contact with a variety of ion-exchange resins, including those used in conventional deionization plants.

EXPERIMENTAL

Samples of water from various sources were extracted four times with redistilled dichloromethane (250 ml water with 4 × 40 ml solvent) and the combined extracts were dried over sodium sulphate. The dried extract was evaporated to 2·5 ml in a Kuderna-Danish flask at 45°C, 800 μl hexane was added, and evaporation was continued to 250 μl. From this solution 5 μl aliquots were injected into a gas chromatograph consisting of a 4 m length of 1·8 mm ID steel column packed with 5% Carbowax 20 M on Diatomite C-AW-DMCS. The carrier gas was argon at a flow rate of 11 ml/min and the column oven was at 150°C. Nitrosamines were detected by a chemiluminescent detector (Thermal Energy Analyzer Model TEA 502, Thermo Electron Corporation, Waltham, Mass., USA) connected to the gas chromatograph. The detector was calibrated for six commonly studied nitrosamines, but will respond to all *N*-nitroso compounds eluting from the chromatograph.

Deionized water is prepared on site using a conventional mixed-bed ion-exchange resin, and samples of the resin were examined for nitrosamines. The resin (50 g) was slurried with distilled tap-water (free from nitrosamines above the 0·005-μg/litre level) and left overnight at room temperature. The resin was filtered off and dichloromethane extracts of the water were treated and analysed as described above. The constituent cation- and anion-exchange resins used for mixed bed deionization (respectively Zeocarb 225 and Deacidite FF, both manufactured by Permutit Co. Ltd., London) were treated separately in the same way. The anion-exchange resins, Deacidite E and G, (Permutit Co. Ltd., London), Amberlite IR45 and IR4B and Amberlyst A21 (Rohm & Haas Co., Philadelphia, USA) were also examined. For water, the detection limit for each nitrosamine was 0·005 μg/litre

and for the resins 0·005 μg/kg. Nitrosamines were identified on the basis of retention time and the detector specificity. For extracts in which the TEA gave a positive result above 0·2 μg/kg. the presence and amount of the nitrosamine was confirmed by combined gas chromatography and high-resolution mass spectrometry for at least one batch of each resin type. The apparatus has been described previously in detail (Gough & Sugden, 1975) and consists of a Pye 104 chromatograph with a short polar packed column in series with an open tubular column coated with polar support. Solvent venting and column bypass facilities are fitted, and the chromatograph is interfaced to an AEI MS902 mass spectrometer, with a silicone membrane separator (Gough & Webb, 1972). Nitrosamines are detected and quantified by monitoring the parent ion of each nitrosamine under high resolution with peak matching against a fluorinated hydrocarbon of precisely known mass.

RESULTS AND DISCUSSION

Samples of tap-water were examined on several occasions and in no case were any volatile nitrosamines detected. Distilled tap-water and the dichloromethane and hexane used in extract preparation were also found to be free from nitrosamines on all occasions. However, trace amounts of DMNA, but no other nitrosamines, were detected in some samples of the deionized water which was prepared from the tap-water (Table 1). Four samples (nos 3–6) were taken in the vicinity of the deionization plant. Positive results were obtained from the water taken immediately after regeneration of the resin bed (samples 3 and 4), but not in the two samples taken at a later date. Deionized water at laboratory outlets (samples 7 and 8), originating from the same plant, was examined on two occasions, including one immediately after regeneration, but no nitrosamines were detected. Water reaches these outlets via a storage tank which would have the effect of diluting any nitrosamines. However by taking water from one such outlet and treating it as in the preparation of food extracts by steam distillation, extraction and concentration (Goodhead & Gough, 1975), positive results were obtained in four out of five cases (samples 9–13). On the basis of the amounts of DMNA found in these samples, the contribution toward the dialkylnitrosamine content of a food extract, and hence the apparent nitrosamine level in the original food, can be calculated. In the present work the mean level of DMNA in the treated water was shown to be 0·03 μg/kg, which is two orders of magnitude lower than the levels commonly encountered in cured meats and is thus of no consequence (Gough *et al.* 1977). However the mean DMNA level in 184 samples of food, excluding cured meat and fish, was found to be 0·05 μg/kg, and a major contribution to this is from the deionized water.

Table 1. *Nitrosamines in laboratory water supplies*

Description of tap-water	Sample no.	Concn of DMNA (μg/litre)
Untreated	1	ND
Distilled	2	ND
Deionized	3	0·01
	4	0·01
	5–8	ND
Deionized, distilled	9	ND
	10–12	0·02
	13	0·06

ND = Not detected above the 0·005 μg/litre level
DMNA = Dimethylnitrosamine

A sample (resin 1a,c; Table 2) of the mixed-bed exchange resins from the plant was found to give rise to 0·6 μg DMNA/kg resin (vacuum-filtered weight), after standing overnight in distilled tap water. Mixed-bed resins for water deionization normally consist of a strong cation and strong anion resin, and other batches of these mixed-bed resins were examined for nitrosamines. In all cases DMNA was found (Table 2). The constituent anion and cation resins of the same batches constituting the mixed beds were examined separately, and far greater amounts of nitrosamines were found in the anion-exchange resin (Deacidite FF). This resin contains a tertiary amino functional group and is manufactured from trimethylamine, whereas the cationic resin (Zeocarb 225) contains the sulphonic acid group. The resin containing the highest level of DMNA (3a) smelt strongly of amines, whereas in the other anion-exchange resins of this group an amine odour was barely detectable.

Table 2. *Nitrosamines in strongly ionic resins, Deacidite FF and Zeocarb 225*

Type of resin	Resin batch no.	Concn of nitrosamine (μg/kg)		
		DMNA	DENA	DPNA
Mixed bed	1a,c	0·6(2)*	ND	ND
Mixed bed (1:1, w/w)	2a,c	0·02	0·02	ND
Anion exchange	2a	0·03	ND	ND
Cation exchange	2c	0·009	ND	ND
Mixed bed (1:1, w/w)	3a,c	65	ND	7·0(ND)*
Anion exchange (in water)	3a	125	ND	18(ND)*
Anion exchange (in dichloromethane)	3a	85(120)*	ND	5·5(ND)*
Cation exchange	3c	ND	ND	ND

DMNA = Dimethylnitrosamine DENA = Diethylnitrosamine DPNA = Dipropylnitrosamine
ND = Not detected above the 0·005 μg/kg level
*Figures in parenthesis are based on mass spectrometry.

Table 3. *Nitrosamines in weak anion-exchange resins*

Type of resin	Resin batch no.	Concn of nitrosamines (μg/kg)		
		DMNA	DENA	DPNA
Deacidite E	4	1·3(2)*	0·2	ND
	5	0·07	ND	ND
Amberlite IR45	6	0·9(1)*	ND	ND
	7	0·05	ND	ND
Amberlite IR4B	8	2·8	ND	ND
Amberlyst A21				
in water	9	3·1(2)*	ND	ND
in dichloromethane		16	ND	ND
Deacidite G	10	0·7(1)*	8·1	ND
	11	0·2	2·2	ND
	12	0·5	3·0(3)*	ND

DMNA = Dimethylnitrosamine DENA = Diethylnitrosamine DPNA = Dipropylnitrosamine
ND = Not detected above the 0·005 μg/kg level
*Figures in parenthesis are based on mass spectrometry.

Table 4. *Nitrosamines in anhydrous technical-grade aliphatic amines*

Amine	Concn of nitrosamines (μg/ml)			
	DMNA	DENA	DPNA	DBNA
Dimethylamine	1(2)	ND	ND	ND
Diethylamine	2(1)	120(100)	ND	ND
Dipropylamine	ND	ND	1·6	ND
Dibutylamine	ND	ND	ND	0·6
Trimethylamine	3	ND	ND	ND

DMNA = Dimethylnitrosamine DENA = Diethylnitrosamine
DPNA = Dipropylnitrosamine DBNA = Dibutylnitrosamine
ND = Not detected above the 5×10^{-5}-μg/ml level

All extracts from this resin gave a positive response for dipropylnitrosamine by TEA, but this finding was not corroborated by mass spectrometry and this nitrosamine was regarded as absent. These observations will be reported in detail elsewhere.

To eliminate the possibility of nitrosamine formation by interaction between the resin constituents and a precursor in the water used for extraction, one resin was treated directly with dichloromethane, and similar levels of nitrosamines were found. Weak anion-exchange resins are prepared from secondary amines or polyamines containing the secondary amino function, and in view of the relative ease of nitrosation of the secondary group, some weak exchangers were analysed for nitrosamines. The results are presented in Table 3, from which it can be seen that DMNA was present in all resin extracts. In addition, DENA occurred in several resins and was an order of magnitude more concentrated than DMNA in those resins containing the diethylamino functional group. One resin described as containing the di-*n*-propylamino group was found to give rise only to DMNA.

The resin Amberlyst A21 is a type for use with non-aqueous systems, and one sample of this was subjected to direct extraction with dichloromethane. The level of DMNA found was similar to that detected after water extraction.

When the anhydrous technical grades of the appropriate secondary and tertiary amines were examined directly, all were found to contain varying amounts of the corresponding nitrosamine (Table 4).

We conclude that it is possible to leach out from anion-exchange resins trace amounts of dialkylnitrosamines, which may lead to contamination of material undergoing ion-exchange treatment. In particular, minute amounts of nitrosamines can occur in deionized water, and this may result in contamination of samples which are being analysed for nitrosamines. The nitrosamines are not formed by interaction of free amines in the resin with precursors in water, but may originate in the amines used to prepare the resins.

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REFERENCES

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.

Fazio, T., White, R. H., Dusold, L. R. & Howard, J. W. (1973). Nitrosopyrrolidine in cooked bacon. *J. Ass. off. analyt. Chem.* **56,** 919.

Fine, D. H., Rufeh, F., Lieb, D. & Rounbehler, D. P. (1975). Description of the thermal energy analyser (TEA) for trace determination of volatile and non-volatile N-nitroso compounds. *Analyt. Chem.* **47,** 1188.

Goodhead, K. & Gough, T. A. (1975). The reliability of a procedure for the determination of nitrosamines in food. *Fd Cosmet. Toxicol.* **13,** 307.

Gough, T. A., Goodhead, K. & Walters, C. L. (1976). Distribution of some volatile nitrosamines in cooked bacon. *J. Sci. Fd Agric.* **27,** 181.

Gough, T. A., McPhail, M. F., Webb, K. S., Wood, B. J. & Coleman, R. F. (1977). An examination of some foodstuffs for the presence of volatile nitrosamines. *J. Sci. Fd. Agric.* **28,** 345.

Gough, T. A. & Sugden, K. (1975). Dual column gas chromatographic system for use in mass spectral determination of nitrosamines. *J. Chromat.* **109,** 265.

Gough, T. A. & Webb, K. S. (1972). The use of a molecular separator in the determination of trace constituents by combined gas chromatography and mass spectrometry. *J. Chromat.* **64,** 201.

Pensabene, J. W., Fiddler, W., Gates, R. A., Fagan, J. C. & Wasserman, A. E. (1974). Effect of frying and other cooking conditions on nitrosopyrrolidine formation in bacon. *J. Fd Sci.* **39,** 314.

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SHORT PAPERS

THE PRESENCE OF DIMETHYL- AND DIETHYL-NITROSAMINES IN DEIONIZED WATER

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Summary—Twenty seven of 42 samples of water exposed to deionizing resins contained from 0·03 to 0·34 ppb (μg/litre) dimethylnitrosamine, as determined by gas–liquid chromatography combined with thermal energy analysis, a detection method claimed to be specific for nitrosamines. Twelve of these 27 samples, mainly from water obtained after resin regeneration, were confirmed as containing dimethylnitrosamine by gas–liquid chromatography–high resolution mass spectrometry. Two samples of water deionized at the Center, were confirmed as containing 0·33 and 0·83 diethylnitrosamine as well as the dimethylnitrosamine. The origin of these nitrosamines is unknown at present.

Introduction

Our confirmation of the presence of 3–5 ppb dimethylnitrosamine (DMNA) in fried bacon made with or without the addition of low concentrations of nitrite (Fiddler, Pensabene, Podebradsky, Doerr & Wasserman, 1975) prompted an extensive investigation of the source of the nitrosamine (NA) in this product. Each of the chemicals and reagents used in the analytical procedure was examined. Deionized water was one of the materials tested, and with the gas–liquid chromatography (GLC)–alkali flame ionization detector (AFID) NAs were not detected. The same reagent components were then re-evaluated using a Thermal Energy Analyser (TEA). This detector is extremely sensitive and is claimed to be specific for nitrosamines (Fine, Rufeh & Gunther, 1973). The application of the GLC–TEA to the detection of apparent NAs in deionized water is described and the results are given below.

Experimental

Water (1·2 litre) was extracted twice with 400-ml portions of methylene chloride; the extracts were combined, dried by passage through anhydrous sodium sulphate and concentrated to 1·0 ml. After quantification by GLC using the TEA detector, the extracts were concentrated to about 0·1 ml for GLC–mass spectrometric (MS) confirmation. The operating conditions for the TEA are similar to those described by Fine & Roundbehler (1975) and Fine, Rufeh, Lieb & Rounbehler (1975). A 5 μl injection of 0·6 ng NA could easily be detected. This was equivalent to 0·01 ppb (μg/litre) with respect to the original water sample. Recovery of 250 ng DMNA added to 1·2 litre of distilled water was consistently in excess of 90%.

*Agricultural Research Service, US Department of Agriculture. Reference to a particular brand or firm name does not constitute an endorsement by the US Department of Agriculture over similar products not mentioned.

DMNA and diethylnitrosamine (DENA) were confirmed by matching the GLC retention times with those of the known NAs by peak-matching the exact mass of the parent ions m/e 74·0480 and 102·0723, respectively. The GLC–MS conditions have been published elsewhere (Kushnir, Feinberg, Pensabene, Piotrowski, Fiddler & Wasserman, 1975; Pensabene, Fiddler, Gates, Fagan & Wasserman, 1974). Approximately 3 ng NA/μl injection were needed for MS confirmation. Nitrosamines are potentially carcinogenic and these compounds should be handled with care.

Results and Discussion

Sixty-two samples of water were analysed for NAs. The results are presented in Table 1 and a typical TEA chromatogram is shown in Fig. 1. Volatile NAs were not detected in distilled water or concentrates of methylene chloride solvent. Of the 20 samples of non-deionized water tested, 0·01 ppb apparent DMNA was detected in two samples and 0·05 ppb DENA was detected in a third sample obtained from a tap at the Center, delivering the municipal water supply. Eleven of 14 samples of Center-deionized water had TEA-detectable levels of apparent NAs. Two of these samples were confirmed by MS as containing 0·15 and 0·20 ppb DMNA and 0·33 and 0·83 ppb DENA. Center water examined immediately after it had passed through exhausted resin contained 0·12 ppb DMNA which was confirmed in one of seven samples tested. However, after the resin was regenerated, 13 of 19 samples contained detectable quantities of DMNA (0·03–0·34 ppb). Of these, ten were confirmed by MS. The Center demineralizer was of the mixed-bed type containing strong anion and cation resins. DMNA was also detected, but not confirmed, in both water samples obtained from a different demineralizing system located in the Center pilot plant. This was a two-bed system containing strong cation and intermediate strength anion resins.

Samples of water were taken from five locations at which water from the Center demineralizer had

Table 1. *Nitrosamines in deionized water*

Type of water sample	Analytical method	No. of samples in which DMNA was detected/total no. of samples	Level of DMNA detected (ppb)	No. of samples in which DENA was detected/total no. of samples	Level of DENA detected (ppb)
Non-deionized	GLC–TEA	2/20	0·01	1/20	0·05
Deionized at the Center	GLC–TEA	11/14	0·05–0·20	2/14	0·33, 0·83
	GLC–MS*	2/11	0·15, 0·20	2/2	0·33 0·83
Deionized at the Center, using (a) exhausted resin	GLC–TEA	1/7	0·12	0/7	ND
	GLC–MS*	1/1	0·12		
(b) regenerated resin	GLC–TEA	13/19	0·03–0·34	0/19	ND
	GLC–MS*	10/13	0·08–0·34		
Deionized in a pilot plant at the Center	GLC–TEA	2/2	0·03, 0·05	0/2	ND

ppb = μg/litre ND = Not detected
*GLC–high-resolution MS was used for confirmation of GLC–TEA results indicating the presence of DMNA/DENA.

passed through further individual deionizing cartridges making the water double-deionized. Seven of 12 water samples thus treated apparently contained 0·03 to 0·31 ppb DMNA, but this was not confirmed by MS because relatively small quantities of water had been extracted, leaving insufficient NA for MS. The cartridges were removed and examined for residual NAs by extracting the resin with methylene chloride. The resin in four of the six cartridges tested contained detectable quantities of DMNA. Two of these were confirmed by MS at concentrations of 1·6 and 23 ppb DMNA based on moist resin.

A number of samples containing concentrations of DMNA insufficient for MS confirmation had a peak with the same retention time as authentic DMNA on three different GLC columns (a 15% Carbowax 20 M-TPA on 60/80 Gas Chrom P in a Varian Aerograph 2700 gas chromatograph interfaced with the TEA, a 6% Silar 10 C on 60/80 Gas Chrom P in a Perkin Elmer 3920, and a Chromosorb P in a Hewlett Packard 5710A). The two latter columns were equipped with N–P or alkali flame ionization detectors. In addition, methylene chloride extracts of several samples containing trace concentrations of apparent DMNA were treated with HBr in glacial acetic acid or transferred into aqueous solutions and exposed to ultraviolet light (365 nm). When the samples were rechecked by GLC–TEA, no apparent NA was present. Both of these treatments are known to cleave the N–NO group of nitrosamines (Eisenbrand & Preussmann, 1970; Sander, 1967). Thus, all available evidence suggests that DMNA was present.

Although 0·03–0·20 ppb DMNA was found in the deionized water at the Center, only the higher level could have made a significant contribution to the low levels of this NA detected and confirmed in occasional samples of bacon prepared without nitrite. Since the water was used to prepare some analytical reagents, sufficient DMNA could have been added to yield up to 5 ppb with respect to the meat sample. This does not necessarily invalidate data reporting low levels of DMNA and DENA.

Although the concentrations found were very low, we are reporting this finding of NAs in water exposed

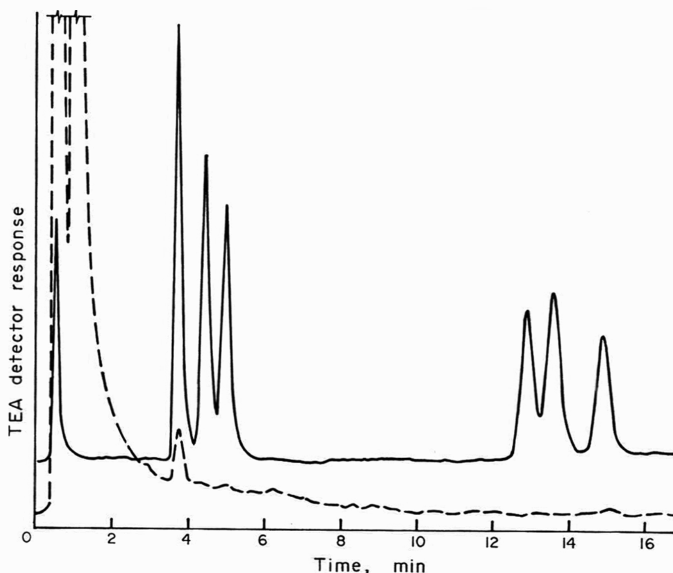

Fig. 1. TEA chromatograms: 2 ng each of standard samples of dimethyl-, methylethyl- and diethylnitrosamine and nitroso derivatives of piperidine, pyrrolidine and morpholine (——); sample of deionized water containing 0·24 ng or 0·03 ppb DMNA (---).

to deionizing resins since it has potentially far-reaching implications in areas such as public health, analysis and toxicology. The origin of these NAs is unknown at present and additional investigations are in progress.

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REFERENCES

Eisenbrand, G. u. Preussmann, R. (1970). Eine neue Methode zur kolorimetrischen Bestimmung von Nitrosaminen nach Spaltung der N-Nitrosogruppe mit Bromwasserstoff in Eisessig. *Arzneimittel-Forsch.* **20**, 1513.

Fiddler, W., Pensabene, J. W., Podebradsky, E. V., Doerr, R. C. & Wasserman, A. E. (1975). Nitrosopyrrolidine and dimethylnitrosamine in pre-fried bacon. 35th Institute of Food Technologists Meeting, Chicago. Ill.

Fine, D. H. & Rounbehler. D. P. (1975). Trace analysis of volatile *N*-nitroso compounds by combined gas chromatography and thermal energy analysis. *J. Chromat.* **109**, 271.

Fine, D. H., Rufeh, F. & Gunther, B. (1973). A group specific procedure for the analysis of both volatile and non-volatile *N*-nitroso compounds in picogram amounts. *Analyt. Lett.* **6**, 731.

Fine, D. H., Rufeh, F., Lieb, D. & Rounbehler, D. P. (1975). Description of the thermal energy analyzer (TEA) for trace determination of volatile and non-volatile *N*-nitroso compounds. *Analyt. Chem.* **47**, 1188.

Kushnir, I., Feinberg, J. I., Pensabene, J. W., Piotrowski, E. G., Fiddler, W. & Wasserman, A. E. (1975). Isolation and identification of nitrosoproline in uncooked bacon. *J. Fd Sci.* **40**, 427.

Pensabene, J. W., Fiddler, W., Gates, R. A., Fagan, J. C. & Wasserman, A. E. (1974). Effect of frying and other cooking conditions on nitrosopyrrolidine formation in bacon. *J. Fd Sci.* **39**, 314.

Sander. J. (1967). Eine Methode zum Nachweis von Nitrosaminen. *Z. physiol. Chem.* **348**, 852.

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SHORT-TERM TOXICITY STUDY OF *PAXILLUS INVOLUTUS* IN THE RAT

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Summary—The toxicity of *Paxillus involutus*, a wild mushroom gathered near the Arctic Circle in Finland, was studied in the rat. When the dried homogenized mushroom in aqueous suspension was administered to male rats by gastric intubation in daily doses of 50 or 250 mg/kg body weight for 40 days, no toxic signs were observed, there being no differences between the test and control groups in general behaviour, body-weight gain, relative weights of the liver, kidney or heart or the gross appearance or histopathology of the major organs.

Introduction

The average yield of wild mushrooms in Finland is estimated to be $1{\cdot}5 \times 10^9$ kg/yr. Although wild mushrooms are rich in minerals and proteins, generally only about one in a thousand of those available are collected. One reason for the low level of utilization of wild mushrooms is the occurrence of some poisonous species.

Paxillus involutus is one of the most abundant of Finnish mushrooms and was apparently widely consumed during World War II as a salted mushroom. However, because this species, which is generally considered to be edible, has been reported to cause poisoning, with some cases of intoxication even proving fatal (Bschor & Mallach, 1963; Schmidt, Hartmann, Würstlin & Deicher, 1971), there has developed a tendency for it to be avoided in Finland. Massive doses of dried *P. involutus* gathered in Poland have induced severe pathological changes in the rat liver and kidney (Lasota & Fortak, 1966), and the present work was designed to study the toxicity in the rat of short-term administration of dried *P. involutus* which had been gathered in Finland. *Boletus edulis*, a delicious species commercially important in Finland, was selected as a control mushroom.

Experimental

The *P. involutus* under investigation was gathered near the Arctic Circle in the autumn of 1975 and was dried for the experiment. *B. edulis* was purchased from a store in packages containing 20 g dried mushroom (dried and packaged by Valio, Finland). An aqueous suspension was prepared from the mushrooms immediately before the administration, using a Potter–Elvehjem glass homogenizer.

Male 3-month-old rats of the Sprague–Dawley strain were divided into four groups of seven. *P. involutus* was given to groups I and II in daily doses of 50 and 250 mg dried mushroom/kg body weight, respectively, dried *B. edulis* was given to group III in daily doses of 250 mg/kg body weight, and group IV (the control group) was given only water. The mushroom homogenate or water was administered to the rats by gavage in a volume of 10 ml/kg once daily for 40 days. The rats were weighed daily throughout the experiment and were given water and food (R3, from Astra-Ewos, Sweden) *ad lib.*

On day 41, the animals were lightly anaesthetized with ether after a night without food and were killed by severance of the aorta. The liver, kidneys and heart were removed and weighed. Samples of these organs and also of the thymus and spleen were fixed in neutral formalin and embedded in paraffin, and 7-μm sections were stained with haematoxylin and eosin. Other organs were studied only macroscopically.

Results and Discussion

The weight gain and general behaviour of the test animals were comparable with those of the control group. There was no change in the relative weights of the livers, kidneys or hearts. Neither macroscopic nor microscopic examination revealed any pathological changes in the test or control groups.

The absence of any pathological changes in rats treated with *P. involutus* in the present study contrasted with the findings of Lasota & Fortak (1966), who previously observed in Poland that massive doses of *P. involutus* killed a rat within 1 wk and caused serious liver and kidney damage. Although the daily dose used in the present study (250 mg/kg) was not high, the administration was relatively long (40 days). In previous studies of poisonous mushrooms we have observed that dried *Amanita virosa* and *Cortinarius speciosissimus* in a single oral dose of 250 mg/kg body weight could induce severe poisoning in the mouse and rat, respectively (Nieminen, Bjondahl, Ojanen, Möttönen, Hirsimäki & Hirsimäki, 1977; Nieminen & Pyy, 1976)

It has been claimed that weather conditions and regions of growth influence the toxicity of mushrooms (Harmaja, Korhonen & Akerblom, 1976). For this reason national investigations of poisonous mushrooms should be accelerated. This is particularly important in the light of the prevailing world food shortage, since mushrooms comprise a large but so far little tapped source of energy.

In view of the well-known marked differences in the response of different species to toxic substances, it is difficult to extrapolate the results of the present rat experiment to man. If it could be determined whether or not similar results are obtained in other species of experimental animals, it would be easier to speculate on the situation in man.

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REFERENCES

Bschor, F. u. Mallach, H. J. (1963). Vergiftungen durch den Kahlen Krempling (Paxillus involutus), eine geniessbare Pilzart. *Arch. Tox.* **20**, 82.

Harmaja, H., Korhonen, M. & Akerblom, H. (1976). *Myrkkysienet ja Sienimyrkytykset.* p.11. Weilin & Göös, Helsinki.

Lasota, W. & Fortak, W. (1966). Studies on Paxillus involutus. II. Changes in some parenchymal organs of young white rats fed with dried Paxillus involutus. *Acta Pol. pharm.* **23**, 81.

Nieminen, L., Bjondahl, K., Ojanen, H., Möttönen, M., Hirsimäki, P. & Hirsimäki, Y. (1977). Sex differences in renal damage induced in the mouse by *Amanita virosa*. *Exp. Pathol.* In press.

Nieminen, L. & Pyy, K. (1976). Sex differences in renal damage induced in the rat by the Finnish mushroom, *Cortinarius speciosissimus*. *Acta path. microbiol. scand.* **84A**, 222.

Schmidt, J., Hartmann, W., Würstlin, A. u. Deicher, H. (1971). Akutes Nierenversagen durch immunhämolytische Anämie nach Genuss des Kahlen Kremplings (Paxillus involutus). *Dt. med. Wschr.* **96**, 1188.

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TERATOGENICITY OF 2,5-DIAMINOTOLUENE, A HAIR-DYE CONSTITUENT, IN MICE

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Summary—Pregnant mice were injected with 2,5-diaminotoluene dihydrochloride, a hair-dye constituent, in a single sc dose of 50 mg/kg body weight on various days of pregnancy, and the foetuses were examined for abnormalities on day 18 of pregnancy. A low incidence of exencephaly and prosoposchisis and a high incidence of skeletal malformations were found in the group treated on day 8. Similar malformations were also detected in two additional groups treated with a higher sc dose (75 mg/kg) or with an ip dose of 50 mg/kg on day 8 of pregnancy. No such malformed foetuses were found in the untreated control group or in the groups treated on days 10–14 of pregnancy, and only a very low incidence of vertebral and rib anomalies followed treatment on day 7 or 9.

Introduction

In recent years, there has been growing concern over the safety of hair dyes. Ames, Kammen & Yamasaki (1975) demonstrated that some hair-dye constituents were mutagenic in bacterial test systems. Searle, Harnden, Venitt & Gyde (1975) also reported that certain hair colourants were mutagenic in bacteria, and the results of their tests in mice indicated some carcinogenic activity. It has been reported that the appearance of dark urine has followed the human use of hair dyes (Marshall & Palmer, 1973; Peter, 1975); since such dyes are used mainly by women, and sometimes during pregnancy, teratogenicity tests of hair dyes should be required. Wernick, Lanman & Fraux (1975) studied the effects of semipermanent-type hair dyes on reproduction and foetal development in rats and rabbits but could detect no evidence of teratogenic effect. Burnett, Goldenthal, Harris, Wazeter, Strausburg, Kapp & Voelker (1976) also reported that five oxidation hair-dye formulations tested were not teratogenic in rats. However, there is too little information in their experiments on several dozen animals to permit conclusions to be drawn about the safety of hair dyes to the millions of human foetuses.

This report concerns the teratogenicity of 2,5-diaminotoluene, which is an essential constituent of conventional oxidative-type hair dyes. This chemical was previously found to be mutagenic in the TA1538 strain of *Salmonella typhimurium* (Ames *et al.* 1975) and has been shown to penetrate human skin during hair dyeing (Kiese & Rauscher, 1968).

Experimental

Material. A hair-dye constituent, 2,5-diaminotoluene dihydrochloride, was obtained from Tokyo Kasei Kogyo Co. Ltd., Tokyo. Assay by gas chromatography showed this material to be 99·9% pure.

Animals and dosage. The animals used were supplied by Nippon CLEA Co. Ltd., Tokyo, from a closed colony of JCL:ddN mice. They were housed at 23 ± 1°C and 55 ± 5% humidity and were given a solid diet of NMF, produced by Oriental Yeast Co. Ltd., Tokyo, and tap-water *ad lib.* Primigravid females, 10 wk or older and weighing 25–30 g, were caged with males of the same strain in pairs overnight, and the next morning females with vaginal plugs were considered to be in day 0 of pregnancy. The pregnant females were divided into eight groups, each of ten or 11 mice, and injected on one of days 7–14 of pregnancy with 2,5-diaminotoluene dihydrochloride, dissolved in distilled water, in a single sc dose of 50 mg/kg (calculated on the treatment-day weight), the maximum tolerated dose for pregnant mice. An untreated control group (13 females) was also maintained. Since the results indicated that 50 mg/kg given on day 8 had the greatest teratogenic effect, the study was extended to include a higher dose of 2,5-diaminotoluene and a different route of administration, an additional two groups of females (ten and 12 mice, respectively) being given either 75 mg/kg sc or an ip dose of 50 mg/kg on day 8 of pregnancy. All mice were killed under ether anaesthesia on day 18 of pregnancy and the uterus was examined for resorption sites. Surviving foetuses were counted, removed, examined for external malformations under a dissecting microscope, weighed and fixed in 95% ethanol. They were then cleared in 1% KOH and stained with Alizarin Red S for demonstration of skeletal abnormalities.

Results and Discussion

The results are summarized in Tables 1 & 2. A fairly low incidence of cranio-facial malformations such as exencephaly and prosoposchisis was found in foetuses from dams treated sc or ip with 2,5-diaminotoluene dihydrochloride on day 8 of pregnancy (Fig. 1a, b). No such cranio-facial malformations have been detected in foetuses of untreated ddN-strain mice examined in this laboratory in the last 8 yr. A high incidence of skeletal malformations, principally fused or distorted thoracic vertebrae associated with absent or fused ribs, was also observed in foetuses

Table 1. *Reproduction and teratology data for mice treated with 2,5-diaminotoluene dihydrochloride in a single sc injection of 50 mg/kg body weight on days 7–14 of pregnancy*

Time of treatment (day of pregnancy)	No. of dams/group	Total no. of			Foetal weight† (g)	No. of		No. of foetuses with			
		Implantations	Dead foetuses or resorptions*	Live foetuses		Ossified vertebrae†	Malformed foetuses‡	AH	E	VRM	P
7	11	126	16 (13)	110	1·3 ± 0·13	36 ± 1·3	3 (3)	1		2	
8	11	119	10 (8)	109	1·3 ± 0·14	36 ± 1·0	20 (18)		3	15	2
9	10	108	10 (9)	98	1·3 ± 0·09	36 ± 1·3	1 (1)			1	
10	10	100	7 (7)	93	1·3 ± 0·18	36 ± 1·6	0				
11	10	105	11 (11)	94	1·4 ± 0·16	36 ± 1·6	0				
12	10	95	11 (12)	84	1·3 ± 0·13	36 ± 1·3	0				
13	10	112	10 (9)	102	1·3 ± 0·14	36 ± 1·5	1 (1)	1			
14	10	89	6 (7)	83	1·3 ± 0·17	36 ± 1·5	0				
Control	13	144	12 (8)	132	1·3 ± 0·12	36 ± 1·2	0				

AH = Abdominal hernia E = Exencephaly VRM = Vertebral and rib malformation P = Prosoposchisis
*Expressed in parentheses as percentage of total implantations.
†Means ± SD.
‡Expressed in parentheses as percentage of live foetuses.

Table 2. *Reproduction and teratology data for mice treated with a single sc or ip dose of 2,5-diaminotoluene dihydrochloride on day 8 of pregnancy*

Dose (mg/kg) and route	No. of dams/group	No. of maternal deaths	Total no. of			Foetal weight† (g)	No. of		No. of foetuses with	
			Implantations	Dead foetuses or resorptions*	Live foetuses		Ossified vertebrae†	Malformed foetuses	E	VRM
75, sc	10	6	34	14 (41)	20	1·4 ± 0·09	36 ± 1·2	7 (35)	1	6
50, ip	12	4	78	40 (51)	38	1·3 ± 0·12	35 ± 0·8	17 (45)	1	16

E = Exencephaly VRM = Vertebral and rib malformations
*Expressed in parentheses as percentage of total implantations.
†Means ± SD.
‡Expressed in parentheses as percentage of live foetuses.

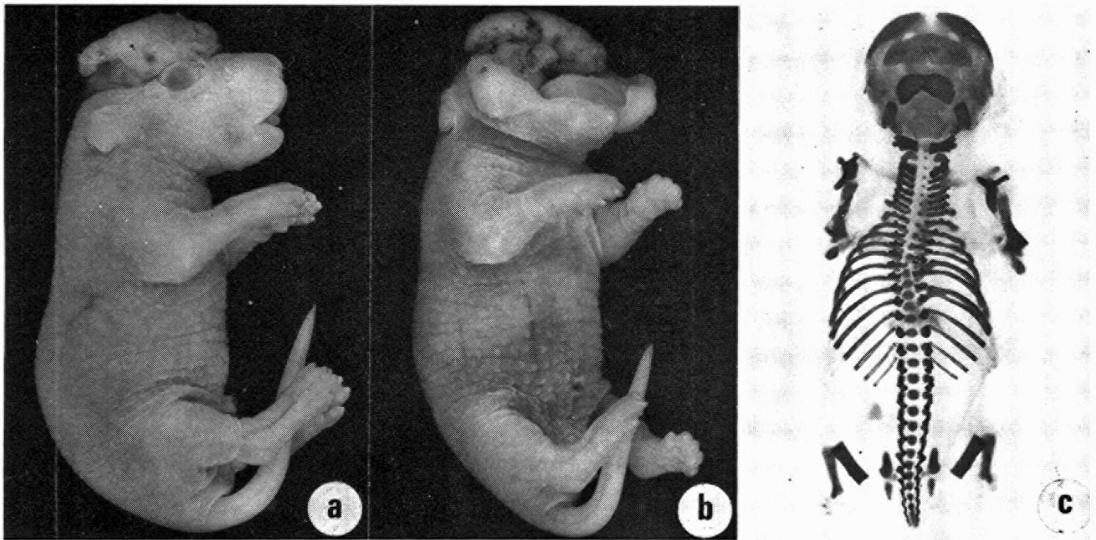

Fig. 1. Malformed foetuses from pregnant mice treated sc with 50 mg 2,5-diaminotoluene dihydrochloride/kg on day 8 of pregnancy, showing (a) exencephaly, (b) prosoposchisis and (c) distorted vertebrae associated with fused ribs.

treated on day 8 of pregnancy (Fig. 1c). Similar vertebral and rib abnormalities were found in a few foetuses in the groups treated on day 7 or 9 of pregnancy. There were no statistically significant differences in the incidence of intrauterine deaths among the groups treated sc with 50 mg/kg on various days of pregnancy or between these and the untreated group, but in groups treated sc with a dose of 75 mg/kg or ip with 50 mg/kg on day 8, some dams died and nearly half of the foetuses died or were resorbed. There were no significant differences in either mean foetal weight or mean number of ossified vertebrae in all eleven treated and untreated groups.

These results suggest that 2,5-diaminotoluene is markedly teratogenic in mice. There were no apparent differences in the types of malformation induced by different doses or by different routes of administration in this series of experiments. The mechanism of this teratogenicity is obscure, and although the 50-mg/kg sc dose was not overtly toxic to the dams, the possibility that it exerted some maternal toxicity and thus affected foetal development indirectly, cannot be altogether dismissed. Nevertheless, the results of Ames *et al.* (1975), showing that 2,5-diaminotoluene was mutagenic in bacteria, suggested that the chemical had the ability to damage cellular DNA, and it is possible, therefore, that the DNA of embryonic cells is involved in the findings reported here. As Connors (1975) reported, numerous DNA-damaging chemicals, such as alkylating agents and electrophilic reactants, have been shown to be teratogenic in mammals, including man in some cases.

Burnett *et al.* (1976) studied five hair-dye formulations containing 2,5-diaminotoluene and found them to be non-teratogenic in rats at dose levels comparable with human exposure. In the present experiment, however, the teratogenicity of 2,5-diaminotoluene was demonstrated at the maximum tolerated dose (50 mg/kg sc) and roughly median lethal doses (75 mg/kg sc and 50 mg/kg ip) for pregnant mice. As Epstein (1973) stated, agents should be tested at dose levels higher than might be anticipated in man following high-level accidental exposure. This is an essential step towards counteracting the insensitivity of conventional test systems, which are based on very small numbers of animals compared with the millions of humans possibly at risk. Kiese & Rauscher (1968) reported that less than 0·2%, or about 4·6 mg, 2,5-diaminotoluene was absorbed through human skin during a standard hair-dyeing procedure. The dose for a 50-kg woman may therefore be calculated as roughly 0·1 mg/kg or 500 times less than the dose (50 mg/kg) shown to be teratogenic in the present experiment. Occasionally, however, human subjects are more sensitive to certain chemicals than are experimental animals. For instance, humans are 60 times more sensitive than mice and 700 times more sensitive than hamsters to thalidomide teratogenicity (Epstein, 1973). Thus, a compound to which man is exposed at levels far lower than the dose that is teratogenic in experimental animals cannot be considered necessarily non-teratogenic in man. The finding that 2,5-diaminotoluene, one of the hair-dye constituents, is teratogenic in mice raises the question, therefore, as to whether there is any risk to the human foetus from maternal use of hair dyes during pregnancy and strongly suggests the need for comprehensive studies on the effect of hair dyes on reproduction.

REFERENCES

Ames, B. N., Kammen, H. O. & Yamasaki, E. (1975). Hair dyes are mutagenic: Identification of a variety of mutagenic ingredients. *Proc. natn. Acad. Sci. U.S.A.* **72,** 2423.

Burnett, C., Goldenthal, E. I., Harris, S. B., Wazeter, F. X., Strausburg, J., Kapp, R. & Voelker, R. (1976). Teratology and percutaneous toxicity studies on hair dyes. *J. Toxicol. envir. Hlth* **1,** 1027.

Connors, T. A. (1975). Cytotoxic agents in teratogenic research. In *Teratology Trends and Applications.* Edited by C. L. Berry and D. E. Poswillo. p. 49. Springer-Verlag, Berlin.

Epstein, S. S. (1973). Environment and teratogenesis. In *Pathobiology of Development—Or Ontogeny Revisited.* Edited by E. V. D. Perrin and M. J. Finegold. p. 105. The Williams & Wilkins Company, Baltimore.

Kiese, M. & Rauscher, E. (1968). The absorption of *p*-toluenediamine through human skin in hair dyeing. *Toxic. appl. Pharmac.* **13,** 325.

Marshall, S. & Palmer, W. S. (1973). Dark urine after hair coloring. *J. Am. med. Ass.* **226,** 1010.

Peter, J. B. (1975). Pigmenturia from "Loving Care" hair dye. *New Engl. J. Med.* **293,** 458.

Searle, C. E., Harnden, D. G., Venitt, S. & Gyde, O. H. B. (1975). Carcinogenicity and mutagenicity tests of some hair colourants and constituents. *Nature, Lond.* **255,** 506.

Wernick, T., Lanman, B. M. & Fraux, J. L. (1975). Chronic toxicity, teratologic, and reproduction studies with hair dyes. *Toxic. appl. Pharmac.* **32,** 450.

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ABSORPTION OF INGESTED TALC BY HAMSTERS

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Summary—To determine talc absorption in the gastro-intestinal tract of hamsters, talc was neutron-activated and suspended in physiological saline and 1-ml aliquots of this suspension were administered by gastric intubation to six 10-wk-old Syrian golden hamsters, which were kept in metabolism cages. The hamsters were killed 24 hr after gavage, and the skinned carcass, gastro-intestinal tract, lungs, liver, kidneys and collected urine and faeces were analysed for ^{60}Co and ^{46}Sc by γ-ray spectrometry. An average of approximately 3 mg talc was found in the tissues and excreta. Of this quantity, 74·5% was found in the faeces, 23·5% in the gut and 1·9% in the carcass. The γ-ray counts from the urine, accounting for 0·09% of the total, were probably due to leaching of ^{60}Co from the talc with subsequent absorption and urinary excretion. This study shows that intestinal absorption of talc is negligible and could have contributed only minimally to the body burden of talc found in hamsters in a previously published inhalation study (Wehner *et al. Fd Cosmet. Toxicol.* 1977, **15**, 121).

Introduction

In a previous study (Wehner, Zwicker, Cannon, Watson & Carlton, 1977a), we investigated the effects of inhaled baby powder (talc), using the Syrian golden hamster as an animal model. In the course of the repeated exposure of these animals to a talc aerosol, unknown quantities of talc passed through their gastro-intestinal tract. This talc originated partly from the ciliated part of the respiratory tract, from where it was brought up by the mucociliary clearance mechanism and swallowed, partly from the pelt, on which some powder was deposited during whole-body exposures and from which it was removed by licking, and partly from the hamster feed, on which some talc powder was deposited during exposures. It was necessary, therefore, to determine in our animal model to what degree the gastro-intestinal tract contributes to the body burden of talc.

Experimental

Neutron activation of talc. Johnson's Baby Powder® (lot 228p), hereafter referred to as talc, was exposed to an integrated neutron flux of about 7×10^{16} n/cm^2, which produced radionuclides by (n, γ) reactions on the major and minor elements in the talc (Wehner, Wilkerson, Cannon, Buschbom & Tanner, 1977b). The neutron-activated talc was allowed to cool for 30 days so that the short-lived neutron-induced isotopes could decay.

Animal treatment. The neutron-activated talc was suspended in physiological saline solution containing 0·6% (w/w) 1% methyl cellulose as the suspending agent. While being agitated by a magnetic stirrer, 1-ml aliquots of the suspension were aspirated with a syringe for administration by gastric intubation to six 10-wk-old female Syrian golden hamsters (*Mesocricetus auratus*, outbred Ela:ENG strain, from Engle Laboratory Animals, Farmersburg, Ind.), maintained in metabolism cages. An additional four (control) hamsters received, also by gastric intubation, 1-ml aliquots of an identical suspension of non-irradiated talc to determine whether the suspending agent or the irradiated talc had a cathartic effect. The hamsters were killed 24 hr after gavage and the skinned carcass, gastro-intestinal tract, lungs, liver, kidneys and collected urine and faeces were analysed for radioactive isotopes by γ-ray spectrometry. The γ-ray counts were compared with those obtained from four control hamsters which had received no talc.

Analysis of samples. Tissue samples for γ-ray counting were preserved either in formalin or by freezing. Small tissues such as the lung and kidney were preserved in formalin, placed under a heat lamp to reduce the volume and then transferred to a 7·4-ml polyethylene vial for analysis. Small frozen-tissue samples and faecal and urine samples were transferred directly to vials for γ-ray counting. Large samples, such as the carcass, required high-temperature ashing to reduce the volume for analysis. Previous work in our laboratory has shown that this does not result in volatilization and consequent loss of the radionuc-

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Table 1. *Distribution of talc, as indicated by γ-ray counts, in tissues and excreta of hamsters 24 hr after administration of a single neutron-activated dose by gavage*

Sample	γ-ray counts expressed as					
	μg talc equivalents			% of total recovered		
	Mean*	SD	SEM	Mean*	SD	SEM
Gastro-intestinal tract	688·4	232·1	94·8	23·5	10·75	4·41
Skinned carcass	56·0	24·8	10·1	1·91	0·73	0·30
Lungs	0·0	—	—	0	—	—
Liver	0·6	0·76	0·31	0·02†	0·02	0·01
Kidneys	1·2	1·19	0·53	0·04†	0·04	0·02
Urine	2·6	0·66	0·33	0·09	0·05	0·03
Faeces	2187	769	314	74·5	11·3	4·61
Total talc accounted for...	2936	746	304			

*Derived from a group of six hamsters. All data are corrected for background and control values.
†Not significantly different from control values at the 0·05 level.

lides considered in this study (L. A. Rancitelli, unpublished data 1976).

The γ-ray analyses were performed on several Ge(Li) and Na(Tl) γ-ray spectrometers. Talc standards in geometrical arrangements identical to those of the samples were counted on each system used for sample analysis. Counting times ranged from 100 to 2000 min, depending on the activity in the samples. The quantity of talc in the tissue sample was determined by comparing the sample with the standards.

Results

The results of this study are shown in Table 1. The γ-ray counts of the hamster tissues and excreta were equivalent on average to a total of 2·94 mg talc. Most (some 98%) of this quantity was found in the faeces (74·5%) and in the gastro-intestinal tract (23·5%). The carcasses accounted for 1·9%. The γ-ray counts of the lungs, kidneys and liver did not differ significantly from those of the controls ($P > 0{\cdot}05$) but the urine counts, although accounting for only 0·09% of the counts, did. No cathartic effect of the suspending agent or of the activated talc was observed.

Table 2 compares the talc data based on the use of ^{46}Sc with that obtained with ^{60}Co for the gastro-intestinal tract, carcass and faeces for each animal. The ratios of ^{60}Co values to ^{46}Sc values are also given and indicate that the ^{46}Sc talc data are in reasonably good agreement with the ^{60}Co data.

Discussion

The isotope ^{60}Co was used to calculate the quantity of talc in all the samples because it has a long half-life (5·2 yr) and easily detectable coincident

Table 2. *Quantities of talc in tissues and faeces of individual hamsters given a single neutron-activated dose by gavage, as calculated from ^{60}Co and from ^{46}Sc data*

Animal no.	Sample	Total talc content (μg)* calculated from counts of ^{60}Co (x)	^{46}Sc (y)	Ratio x/y ± SD
1	GI tract	944	1007	0·94 ± 0·051
	Carcass	67·6	57·8	1·17 ± 0·077
	Faeces	1332	1612	0·83 ± 0·050
2	GI tract	294	271	1·08 ± 0·115
	Carcass	44·2	51·4	0·86 ± 0·062
	Faeces	2232	2346	0·95 ± 0·012
3	GI tract	615	730	0·84 ± 0·080
	Carcass	25·9	27·4	0·94 ± 0·151
	Faeces	2007	2366	0·85 ± 0·015
4	GI tract	869	933	0·93 ± 0·080
	Carcass	96·6	104·2	0·93 ± 0·077
	Faeces	2637	2980	0·88 ± 0·014
5	GI tract	633	663	0·95 ± 0·064
	Carcass	60·9	65·3	0·93 ± 0·087
	Faeces	3417	3540	0·96 ± 0·014
6	GI tract	775	858	0·90 ± 0·054
	Carcass	41·0	36·4	1·13 ± 0·121
	Faeces	1495	1666	0·90 ± 0·059

GI tract = Gastro-intestinal tract
*Corrected for control values.

γ-rays. In addition, ^{46}Sc activity was used to estimate the quantity of talc in a number of samples to permit comparison of the sets of talc values obtained with the ^{46}Sc tracer and with the ^{60}Co tracer. The advantage of using a pair of radioactive tracers to monitor the transfer of talc lies in observing two elements with different dissolution and transfer coefficients in biological systems. If the ^{60}Co data and the ^{46}Sc data lead to similar talc values, it can be concluded with a high degree of probability that the radionuclides were transferred together by particulates. An alternative situation producing the same ^{60}Co-to-^{46}Sc ratio in the tissues as was observed in the talc would require these radionuclides to be leached and transferred in identical fashion. The latter possibility is not supported by past experience in our laboratory, which has suggested that biological systems take up Co more readily than Sc (L. A. Rancitelli, unpublished data 1976). To obtain additional information on the degree of radionuclide leaching from talc in body fluids, a leaching study was undertaken in which neutron-activated talc was suspended in bovine serum and in dilute 2N-HCl for various periods of time (Wilkerson, Wehner & Rancitelli, 1977).

A suspending agent was used to prevent rapid sedimentation of the talc particles in the vessel containing the suspension and in the syringe during gavage. Total quantities of talc (mean $\pm$ SEM = 2·94 $\pm$ 0·30 mg) found in the hamsters and their excreta were reasonably uniform.

Most of the talc was recovered in the faeces and gastro-intestinal tract of the hamsters, as was expected. The data show that 24 hr after gavage approximately 75% of the talc had been excreted in the faeces while approximately 23% was retained in the gastro-intestinal tract. Additional residence time in the gut prior to complete elimination of talc-containing faecal matter would probably have resulted in only an insignificant increase in talc absorption, since the passage of 75% of the talc through the gastro-intestinal tract and retention of 23% within it for 24 hr resulted in only 2% absorption. It appears reasonable, therefore, to assume that additional time until complete elimination of the talc from the gastro-intestinal tract would have contributed not more than a small fraction of 1% to the total level of absorption. The zero values for the lung indicate that none of the administered suspension was aspirated by the animals and that no translocation of talc to the lungs took place.

The results of the leaching study mentioned above (Wilkerson *et al.*, 1977) showed that suspending neutron-activated talc in 2N-HCl removed from 0·5 to 1% of ^{60}Co and 5% of ^{46}Sc from the talc, primarily by leaching. This suggests that leaching of radionuclides from talc may have taken place in the acid environment of the stomach of the gavaged hamsters. Since, according to measurements in our laboratory, this particular talc contained 64 ppm Co, and since 2·94 mg talc passed through the gastro-intestinal tract of the hamsters, 0·5% leaching would have removed from the talc and made available for absorption about 940 pg Co. On the basis of a suggested figure of 30% for absorption from the intestinal tract (ICRP Committee II, 1960), this would have resulted in the translocation of about 300 pg Co to the blood. If leached Co which translocates to the blood is largely excreted in the urine, the excreted ^{60}Co should be measurable in the latter fluid. If 300 pg Co were excreted in the urine, the resulting urinary γ-counts from ^{60}Co would be equivalent to roughly 4 μg talc. Since the measured urine counts were equivalent to 2·6 μg talc (Table 1), leaching of ^{60}Co in the acid environment of the stomach with subsequent absorption from the intestinal tract and excretion in the urine could well account for the quantities of ^{60}Co found in the urine.

Apparently 1·9% of the talc passing through the gastro-intestinal tract was absorbed and translocated to parts of the body other than the lungs, liver, kidneys, and gut. Further differentiation of translocation sites was beyond the scope of this investigation.

From the results of this study, it can be concluded that intestinal absorption contributed only minimally to the body burden of talc demonstrated in the hamsters in our previously conducted inhalation study (Wehner *et al.*, 1977a).

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REFERENCES

ICRP Committee II (1960). Permissible dose for internal radiation. *Hlth Phys.* **3**, 161.

Wehner, A. P., Zwicker, G. M., Cannon, W. C., Watson, C. R. & Carlton, W. W. (1977a). Inhalation of talc baby powder by hamsters. *Fd Cosmet. Toxicol.* **15**, 121.

Wehner, A. P., Wilkerson, C. L., Cannon, W. C., Buschbom, R. L. & Tanner, T. M. (1977b). Pulmonary deposition, translocation and clearance of inhaled neutron-activated talc in hamsters. *Fd Cosmet. Toxicol.* **15**, 211.

Wilkerson, C. L., Wehner, A. P. & Rancitelli, L. A. (1977). Leaching of radionuclides from neutron-activated talc in serum and in dilute hydrochloric acid. *Fd Cosmet. Toxicol.* **15**. In press.

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Review Section

CRITERIA EMPLOYED BY THE EXPERT PANEL OF FEMA FOR THE GRAS EVALUATION OF FLAVOURING SUBSTANCES

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Summary—An updated account is presented of the activities of the Expert Panel of the Flavor and Extract Manufacturers' Association for establishing the generally recognized as safe (GRAS) status of flavouring substances in accordance with the Food Additives Amendment to the US Federal Food, Drug, and Cosmetic Act. The procedure entails the development and application of criteria for the safety evaluation of flavouring substances on the basis of data concerning chemical composition and structure, metabolism, toxicology and usage levels as reported by industry. The 'GRAS lists' emanating from these judgements of the Expert Panel have been the subject of eight papers published during the period 1960–1976.

Introduction

The statutory definition of the term 'food additive' in the Federal Food, Drug, and Cosmetic Act exempts any substance "generally recognized, among experts qualified by scientific training and experience to evaluate its safety, as having been adequately shown through scientific procedures (or, in the case of a substance used in food prior to January 1, 1958, through either scientific procedures or experience based on common use in food) to be safe under the conditions of its intended use". This provision has come to be designated by the acronym 'GRAS'. It should be emphasized that recognition of safety rests on the opinion of qualified experts, not limited to those in the regulatory agency, and relates specifically to the conditions of use of the substance in food.

In response to the Food Additives Amendment, the Flavor and Extract Manufacturers' Association (FEMA) organized in 1960 a Panel of expert scientists, not affiliated to the flavour industry but with special competence in toxicology, pharmacology and biochemistry, to evaluate the 'GRAS status' of the many hundreds of organic chemicals contained in natural and artificial foods and flavours (Hall & Oser, 1961). The Food and Drug Administration was kept informed of this activity of FEMA from its very inception and, in fact, recommended that a large series of natural substances be included in the Panel's evaluations. Subsequent to the release in 1965 of the first GRAS list of some 1100 flavouring substances and their use levels (Hall & Oser, 1965), the FDA adopted virtually the entire list in the form of two regulations (21 CFR 121.1163 and .1164) covering, respectively, natural and synthetic flavouring substances.

It may be noted that "scientific procedures" as defined in the original regulations included "not only original animal, analytical, and other scientific studies, but also an unprejudiced compilation of reliable information, both favorable and unfavorable, drawn from the scientific literature" (21 CFR 121.1 (h)). It was later proposed to modify this definition to include "those human, animal, analytical, and other scientific studies, whether published or unpublished, appropriate to establish the safety of a substance" (*Federal Register* 1974, **39** (Part II), 34195).

It is within the spirit of these definitions that the Panel has evaluated the safety of flavouring substances, the vast majority of which have been in wide use for many years and are generally regarded as normal components of food. The results of these studies have been reported in a series of papers in *Food Technology* (Hall & Oser, 1965 & 1970; Oser & Ford 1973a,b, 1974, 1975 & 1977; Oser & Hall, 1972) and additional papers have appeared on this subject elsewhere (Bednarczyk, Galetto, Hall & Stahl, 1975; Gerarde, 1973; Hall & Oser, 1968; Oser, 1970).

The principles and rationale adopted in evaluating the safety of present or proposed flavouring substances to determine whether or not such ingredients can be regarded as GRAS have been described in the above cited reports and summarized in a presentation by the late Dr. Horace W. Gerarde (1973), a member of the Panel. The objectives of this paper are to review the policies and criteria that have been employed by the Panel, taking the following factors into account: the changing patterns and levels of use of individual flavouring substances, including increases or decreases in total usage as indicated by

estimates of annual poundage; new and more reliable information on the daily consumption of foods by categories and population groups, the data for which were accumulated by the National Academy of Sciences–National Research Council (Food Protection Committee, 1972) under contract with the FDA; current scientific literature reviews authorized and projected by the Food and Drug Administration and concerned with both GRAS and regulated food additives; an updated industrial survey of the usage of flavouring substances, sponsored by FEMA; advances in analytical methodology as related to the detection of impurities and the identification of the constituents of natural products; improvements in toxicological and metabolic procedures; and appropriate adaptation of the Panel's standards to meet these changing circumstances.

The Panel felt it important to view flavouring substances in perspective against the totality of environmental hazards and to assign suitable priorities for the toxicological assessment of flavourings. As stated in the NAS–NRC (1975) report (p. 112). "To provide optimum assurance of public safety within the existing limitations of capabilities available, toxicological facilities and skills for evaluating safety must be concentrated on environmental situations in which there is a reasonable expectation that exposure to chemicals may cause real hazards. It is clear that materials being widely distributed into the environment so as to place a large segment of the population at risk would be assigned a high priority for risk–benefit assessment, including toxicity tests. Conversely, those materials which are encountered in trace quantities by a limited population or even a large population would be given a lower priority. It is equally clear that, if the trace quantity is well below the known level of toxic effect, an even lower priority may be assigned."

Particularly relevant to flavouring substances is the statement by the Food Protection Committee (1969) of the NAS–NRC, republished as an Appendix to a later monograph (NAS–NRC, 1970), that "Because of the thousands of natural and synthetic substances present in food at low and intermediate levels, it becomes necessary to establish a reasonable system of priorities for the further study of those substances not yet fully evaluated. To study every chemical to the same extent as those that must be used at close to their safe levels would represent an unjustifiable expenditure of effort not contributing significantly to protection of public health. It is neither practicable nor necessary to undertake experimental toxicological studies of every chemical to which man is exposed; to do so would be to assign equal importance to problems of unequal risk. This would deny the value of experience in assessing probable risk. All environmental exposures must be subjected to scientific evaluation, but not all exposures require experimental toxicological study."

Criteria for evaluation of the safety of flavouring substances

The NAS–NRC (1970) report on Evaluating the Safety of Food Chemicals relates to food additives in the broadest sense rather than to the more specific category of flavouring substances, which are unique in that they are almost always used at extremely low levels and are inherently self-limiting by reason of their intense sensory properties.

The evaluation of the safety of flavourings, as practised by the Panel, involves the simultaneous application of a series of criteria, every one of which must be considered in arriving at a determination of safety-in-use. No single criterion is used to the exclusion of others or is expected to require absolute, independent justification. Instead, all must be mutually supportive and unanimously agreed upon for a conclusion on safety to be reached. Briefly, these criteria are chemical identity, structure and purity, natural occurrence of the chemical in food, concentration of the chemical in food and in the total diet, toxicological evidence in animals and man and metabolic fate in mammals.

It is obvious that some of these requirements are not quantifiable or subject to numerical rating, nor is it practical to assign relative weights for objective appraisal. Hence, a Panel decision must be regarded as a value judgement taking into account all relevant factors. From the beginning of its operations, the Panel has considered each flavouring substance on an individual substance-by-substance basis with due regard to other compounds related by chemical structure or biological properties.

Identity of the substance. The natural substances reviewed by the Panel were proposed for consideration by the Food and Drug Administration. They included essential oils, oleoresins, extracts and distillates as sold commercially and commonly used in food flavouring. Descriptions of these products are found in various text books and reference books (Arctander, 1960 & 1969; *Fenaroli's Handbook of Flavor Ingredients*, 1975; *Food Chemicals Codex*, 1972; Guenther, 1948–1952; *United States Dispensatory*, 1967).

Flavouring substances were assumed to have the identity and structure implicit in their chemical names, irrespective of whether they were produced synthetically or isolated from natural sources. Account was taken of the chemical and physical specifications, source, identity, composition and purity of the flavouring compounds as given in the above-mentioned compendia.

Where any impurity of toxicological significance was indicated or suspected, either because of the natural origin of the substance or its method of isolation or synthesis, no opinion was expressed until further information had resolved the question of the relevance of the impurities to safety considerations. In this connexion, special attention was paid to mixtures of optical or steric isomers.

Other considerations of structural analogy applicable to the toxicological evaluation of categories of compounds are discussed below.

Natural occurrence in food. A report by a WHO Scientific Group (1967) makes the point (p.7) that: "It has been stated that all intentional food additives should be subjected to individual toxicological investigation. This generalization needs modification along the following lines: (a) Many additives may already be present in food or elsewhere in the environment. The background occurrence of the chemical must be taken into account in the evaluation of its safety..."

Foods as produced and those consumed in their natural form enjoy the presumption of safety if no adverse effects have been observed as a result of their ingestion. The misconceptions concerning natural occurrence range from the mythical dogma of the 'natural food' faddist to disbelief in the value of human experience. Ironically, however, knowledge gained from experience is used in some way by virtually everyone, including those who most vehemently deny its validity.

The toxicity of a substance is its capacity to cause injury whereas 'safety' is a negative concept and can only be defined in terms of the virtual absence of injury or harm, in whatever form, under conditions of normal or predictable use. 'Harm' is a positive concept and is generally observable in some way. The term 'hazard' is defined as the probability that harm could occur under the conditions of use.

Several excellent publications (American Chemical Society, 1969; Central Institute for Nutrition and Food Research, 1973; Food Protection Committee, 1973; Gontzea & Sutzescu, 1968; Liener, 1969) list or discuss thousands of chemical substances present as natural constituents of food. The fact that through the years man has ingested natural foods, with their multiplicity of chemical components, without apparent harm does not by itself constitute 'proof' of safety. In fact "it is toxicologically axiomatic that if almost any one of this myriad of chemical substances were tested in experimental animals by today's standards of safety evaluation, it would be shown to be toxic. Thus, it may be said that in the natural foods of our everyday diet there are thousands of toxic substances. This does not imply, however, that a hazard exists in this situation.....Viewing all chemicals that are present in our food supply—the natural components, agricultural chemicals, food additives, and natural and man-made contaminants—in perspective, it is clear that the greatest area of the unknown involves the natural and normal components of our foods" (J. M. Coon, cited from Food Protection Committee, 1973).

The ease, even the possibility, of establishing a causal relationship between human experience and potentially hazardous environmental factors is in inverse proportion to the time required for adverse effects to develop and be observed. Botulism, shellfish poisoning and cyanide toxicosis from nuts or kernels are acutely manifested reactions, easily associable with their cause, but the same cannot be said for toxic reactions following long periods of latency, such as carcinogenicity or mutagenicity.

Nevertheless, one must not ignore the value of experience based on exposure of the species with which we are most concerned, namely man. The Panel takes the position that if there is no basis for suspicion and, in addition, there exists other evidence of safety based upon considerations of structure, metabolic fate, toxicological data or low use levels, natural occurrence in foods has important supportive value.

There are frequently borderline instances where a substance not known to occur naturally in foods has a structure so similar to others already discovered in food that it ultimately yields to the scrutiny of the analytical chemist and becomes identified in food.

More pertinent to the present discussion than the variety of toxic substances in natural foods is the large number of chemicals that have been found to comprise the flavour and taste component of natural foods, thanks to the application of modern procedures of gas chromatography, mass spectroscopy, nuclear magnetic resonance, and other sophisticated analytical methods. Nowhere is this better illustrated than in the compilation published by the Central Institute for Nutrition and Food Research (1973, sup-

Table 1. *Volatile compounds identified in natural foods*

Type of food	No. of different compounds found								
	Hydrocarbons	Alcohols	Carbonyls	Acids	Esters	Bases	Sulphur compounds	Miscellaneous	Total
Coffee	52	18	104	22	37	100	73	102	508
Tea	27	41	105	25	63	52	12	50	375
Cocoa	45	28	58	41	63	92	15	69	411
Milk	43	16	61	20	48	3	10	43	244
Cheese (cheddar)	32	11	25	27	25	14	6	12	152
Bread	2	15	63	15	10	41	4	10	160
Beef, chicken and turkey, raw	—	2	23	2	9	4	7	3	50
Beef, cooked	44	26	74	19	20	38	64	25	310
Fish	10	3	42	5		19	7	4	90
Citrus fruits	70	64	62	17	62	1	3	45	324
Apples	8	39	39	49	80	4	—	10	229
Raspberries	4	38	37	14	38	—	—	8	139
Strawberries	19	48	28	30	105	—	4	16	250
Potatoes	24	24	34	13	1	9	21	6	132
Carrots	30	10	27	1	—	2	3	5	78
Tomatoes	17	36	68	12	27	4	8	22	194
Onions and garlic	1	5	18	2	5	4	66	3	104

Adapted from "Lists of Volatile Compounds in Food" (Central Institute for Nutrition and Food Research. 1973).

plemented through May 1975) of the Netherlands. For some 87 different foods, this lists in seven defined chemical categories, plus a miscellaneous one, 2600 compounds that have been identified, and gives references to the pertinent literature. Table 1 derived from this publication shows for each chemical category the numbers of compounds that have been found in a cross-section of foods and beverages.

Significance of structure. Structural characteristics and inter-relationships among various classes of compounds are most important factors in predicting the metabolic fate and potential toxicity of ingested chemicals. That this is widely acknowledged is indicated by the following quotation from the recent monograph of the NAS–NRC (1975);

> "Many important decisions, at least about the sequence of testing, can be made without testing at all on the basis of analogies with other known chemicals. Structure–activity relations are reasonably well understood for some groups of chemicals and some toxic effects, less well-known for others. However, many new industrial chemicals differ only trivially from other known materials and relatively few fall into genuinely unknown groups. Those that do will require correspondingly more complex testing."

In applying this criterion to flavouring substances, three classes may be considered, each with particular regard to the levels and conditions of use, as follows:

(I) Compounds of simple organic structure that are readily handled through known metabolic pathways and without adverse biochemical, physiological or pharmacological effect.

(II) Compounds structurally analogous to those in class I and whose metabolic fate can also reasonably be assumed not to be associated with adverse biochemical, physiological or pharmacological effect.

(III) Compounds with structures so different from those in classes I and II that reasonable assumptions regarding metabolic fate and freedom from the possibility of adverse effect are precluded.

Stemming from these considerations is a rationale by which information on substances of related chemical structure and known or assumed metabolic fate may be brought to bear in assessing the safety of each individual substance. The Panel is aware that because of some exceptions to this rule it must be applied judiciously.

At this point it should be noted that flavour chemicals made synthetically correspond structurally in virtually all cases to their naturally occurring analogues. The great majority fall into classes I and II. These include simple aliphatic alcohols, aldehydes, acids and esters, whose biotransformations are well understood. Also in this category are mononuclear aromatic compounds containing only *C*, *H*, and *O*, with simple alkyl, hydroxyl, aldehyde, carbonyl, carboxyl or ester substitution. These have been the subject of numerous research papers and biochemical texts (Ambrose & Sherwin, 1933; Handler & Perlzweig, 1945; Hathway, 1970, 1972 & 1975; Parke, 1968; Roe, 1970; Sherwin, 1922; Stekol, 1941; Williams, 1959).

In general, metabolic pathways and toxicity are determined by the functional groups attached to a molecule. If the functional groups undergo successive transitions, as occur with hydroxyl, aldehyde and carboxyl groups in straight-chain aliphatic compounds, other compounds of similar basic structure are regarded as metabolized in the same way. On an aromatic ring, such groups act as 'metabolic handles' which determine the direction of biotransformation and detoxication.

The toxicity of a compound in a homologous series can frequently be predicted from that of the adjacent members. The intensity and potency of the biological activity of a chemical are related to the size of the molecule and the manner in which the functional or structural features influence metabolism. The introduction of double or triple bonds in a hydrocarbon chain tends to increase its activity above that of the corresponding saturated molecule. Alicyclic compounds are generally less toxic than their aromatic analogues.

A large proportion of the chemical entities in class I are either naturally occurring food flavourings, metabolites of major food components (e.g. carbohydrates or fats) or components of normal body fluids or tissues (e.g. constituents of the Krebs cycle). None of these facts alone establishes the safety of such substances, but when considered together with other evidence, they contribute to the totality of information upon which safety evaluations may be made.

Substances falling into class II are assumed to be metabolized in a manner similar to their close structural analogues in class I. Even where direct experimental evidence itself is lacking, a presumption of safe biotransformation at low levels of intake based largely on reasoning by analogy appears fully justified. Substances in class II may be encountered less commonly in foods or may belong to small groups of food components, such as certain hydrocarbon structures which are branched or include an unusual conjugated or unconjugated substituent. Many alicyclic compounds and some terpenoid structures fall into this category, as do some mononuclear aromatic compounds with generally innocuous functional groups.

Class III comprises chemicals with structures so unusual or complex that a judgement of metabolic fate would be difficult in the absence of specific supporting evidence. Examples are extensively branched compounds, containing certain unsaturated ether or epoxy groups, and complex polycyclic or heterocyclic compounds. These xenobiotics are less likely to be encountered in natural foods or physiological tissues. However, more and more hitherto exotic compounds are being discovered in food through the use of modern analytical procedures, notably gas chromatography and mass spectroscopy. Also included in this class would be any substance considered to be analogous to one that had been found to induce serious adverse effects, or that could reasonably be expected to do so. Examples of flavouring compounds that fall into class III have been subjected to toxicological testing on the recommendation of the Panel (Oser, Carson & Oser, 1965).

Predictions of the metabolic pathways of flavouring substances are based on a large background of biochemical information obtained for the most part at dosages substantially higher than dietary levels. When toxic effects occur, they are generally the result of

a degree of exposure exceeding the capacity of the organism's 'normal' or preferred routes of detoxication and elimination, with consequent accumulation of the substance or its usual metabolites, the production of more toxic metabolites or the evocation of alternative pathways. This is discussed more fully below in relation to chemical structure and the extrapolation of high-dose responses in animals.

Intake levels and priorities. In the early stages of its deliberations, the Panel was presented with data on the usual and maximum levels of use of flavouring substances incorporated during the commercial production of the major categories of foods (i.e. baked goods, beverages, frozen desserts, confectionery and so on). However, since there was no reliable information on the actual consumption of foods in these categories, grossly exaggerated estimates of total intake were based on arbitrary estimates of portion sizes, and on the assumption that all foods flavoured with the substance in question were eaten daily. Moreover, the use of a given flavouring material in lady-fingers, for example, was extrapolated to an assumed occurrence in the entire category of 'baked goods'. As a result, calculations of the total intake of any given flavouring substance often exceeded by far its total annual production.

The obvious need for more valid estimates of food consumption within each category has been met in part by several relatively recent surveys, particularly those conducted under the sponsorship of the Food and Drug Administration by the NAS–NRC on the consumption patterns of some 48 categories of foods, and by Market Research Corp. of America (MRCA) on the frequency of consumption of specific food items (Food Protection Committee, 1972).

These and related publications provide estimates of intake of flavouring materials considerably below those originally assumed for the purpose of the Panel's calculations. The NAS–NRC GRAS Review Committee acknowledged making a series of assumptions which involved cumulative exaggerations, such that their estimates were larger than the actual daily intakes by one to several orders of magnitude. Nevertheless the data show average intakes of flavouring substances to be at very low levels.

The NAS Committee realized that particularly for certain non-flavouring additives, which could be the possible focus of interest, there was a need for still more refined estimates which could also reflect the distribution of intakes throughout the population. Such a method has been devised (Hall, 1976) and has now been applied to 12 selected flavouring substances chosen to cover a wide range of possible intakes and patterns of usage. A comparison of the estimated possible average daily intakes (PADI) from the 1972 publications of the NAS–NRC GRAS Survey and the mean intakes from total observations (MITO) from the new refined method is shown in Table 2. While a critique of the new method is beyond the scope of this paper, it is quite clear that it provides estimates of mean intake which are much closer to *per capita* intakes, independently calculated from the poundage used by the industry, than were the previously used PADI values.

The new method permits estimates not only of the mean intakes, but also of the intake by any specified percentile of the population. We have examined the relationship between the mean and the 99th percentile for the 12 flavourings and 11 other substances so far subjected to this method. The 99th percentile is a low multiple (<10×) of the mean when the number of pounds used is large or when the ingredient is used in many different foods. If the usage in pounds is small, or the substance is used in only a few foods,

Table 2. *Comparison of NAS–NRC estimates of possible average daily intakes (PADI), mean intakes from total observations (MITO) calculated by refined method and* per capita *(CAP) intakes based on pounds used*

Substance	FEMA no.	Estimates of daily intake (mg)			PADI/MITO	CAP/MITO*
		PADI	MITO	CAP		
α-Ionone	2594	0·96	0·019	0·12	51	6·3
2-Hexenyl acetate	2564	1·48	0·0010	0·0015	1500	1·5
Allyl disulphide	2028	2·18	0·000072	0·0014	30.000	19
4-(*p*-Hydroxyphenyl) butan-2-one	2588	2.69	0·034	0·0089	79	0·26
Allyl cyclohexane butyrate	2024	3·41	0·0012	0·00035	2800	0·29
Eugenol	2467	6·99	0·020	0·60	350	30
Menthol	2665	13·4	0·043	0·96	310	22
Ethyl methyl phenyl glycidate	2444	22·5	0·210	0·22	110	1·05
Maltol	2656	29·2	0·87	0·38	34	0·44
Methyl salicylate	2745	37·1	0·63	6·6	59	10
Cinnamaldehyde ethylene glycol acetal	2287	100	0·00081	0·00079	123.000	0·98
Black pepper oleoresin	2846	289	2·24	2·0	130	0·89

*Discrepancies between the *per capita* estimates (from pounds used) and the MITO estimates (new method) may arise from (i) errors in the poundage figures on which the *per capita* estimates are based; (ii) diversion of the ingredient to non-food use; (iii) unexpected use of the synthetic to replace an equivalent natural product; (iv) errors in the application of the new method.

Table 3. *Classification of 1428 flavouring substances according to* per capita *intakes and 20 times the* per capita *intakes*

Intake (mg/day)	No. of flavourings/intake group: Based on annual poundage	Based on annual poundage × 20*
> 100	3	47
10–100	25	79
1–10	68	136
0·1–1·0	118	220
0·01–0·1	189	421
0·001–0·01	348	450
0·0001–0·001	477	75
0·00001–0·0001	200	0†

*Estimate of 99 percentile intake level.
†Requires annual volume of < 1lb.

the 99th percentile may be a higher multiple (× 20–30) of the mean, reflecting the consequences of unusual patterns of food consumption.

This method confirms the usefulness of *per capita* data as an approximation of mean intakes. It also shows that 20 times the mean (or *per capita*) intake is generally an overestimate of 'high' intake, corresponding to at least the 90th percentile, and frequently to more than the 99th. Table 3 displays the numbers of flavouring substances at various intake levels on a 'mean' (*per capita*) and 'high' (20 × *per capita*) basis.

One point relevant to many flavouring ingredients concerns processing losses—especially those caused by volatilization. Commercially heat-processed foods such as 'boiled' candies and baked goods, as well as the products of institutional and home cooking, are subject to large evaporative losses of volatile flavour components. As a guide to the levels of volatile flavourings that may remain after heat processing, one may use their concentrations in foods that are not heat-processed, such as beverages and frozen desserts.

While structure and metabolic fate are usually the primary factors considered in safety evaluation, a decisive role is that played by the levels of intake that might reasonably be expected to be consumed by any identifiable portion of the population. Like most toxicologists, the Panel subscribes to the oft-quoted dictum of Paracelsus "*Sola dosis facit venenum*" (only the dose makes a poison) which may be expressed in modern terms as: "There is a safe dose and an unsafe dose for any chemical, regardless of its chemical structure" (Gerarde, 1973).

Because of the need to establish priorities, the Panel decided to set certain conditions which, assuming all were satisfied, would justify the conclusion that the use of a given flavouring agent presented no significant toxicological hazard. The Panel took the position that "flavor ingredients used at average maximum levels of less than 10 ppm in foods, and at an annual volume of less than 1,000 pounds, for at least 10 years of common use, can indeed be said to be toxicologically inconsequential, *provided* the chemical structure is compatible with a reasonable presumption of the capacity of the body to dispose of trace quantities safely through known metabolic pathways" (Hall & Oser, 1968).

These conditions are consonant with the guidelines later recommended by the Food Protection Committee for estimating toxicologically insignificant levels of chemicals in food (Food Protection Committee, 1969). These guidelines included the proviso that "Chemicals that exert significant biological effects [intended to refer to pesticides, drugs and known carcinogens—B.L.O./R.L.H.] and that are useful or unavoidable in food or any other part of the environment will continue to be subjected to laboratory investigations to establish safe levels....."

A 1971 survey of 1499 flavouring ingredients showed that 75% were used in foods at levels not exceeding 100 ppm. Furthermore, the annual food usage of 76% of GRAS flavouring substances was less than 1000 lb (a consumption on a *per capita* basis approximately equivalent to 0·1 μg/kg body weight/day).

Subsequently, the Panel dropped the term "toxicologically insignificant usage" (TIU) which was the subject of some misinterpretation and adopted instead "priority low—usage minimal" (PLUM) which more clearly indicates its purpose. In place of setting a maximum level in foods, PLUM relates the maximum intake of a flavouring substance to both food consumption data and average levels of use in each food category. The highly exaggerated assumption is made that all foods containing the flavouring substance are eaten every day. For PLUM classification all the following criteria must be satisfied: the substance must be of simple structure and of known composition and purity; its structure must suggest that it will be readily handled through known metabolic pathways and will be excreted without toxic effect; it must be a member of a structurally related group that, without known exception, is or can be presumed to be of low toxicity; it must not be used at levels that would result in an average daily intake of more than 2·0 mg.

Metabolic fate. Given the chemical structure of a flavouring substance, available information concerning its metabolism and the biotransformations it may undergo play a predominant role in assessing its potential toxicity. The classification of chemicals by structure into three classes (p. 460 is predicated on prior consideration of their metabolic fate which, in the broadest sense encompasses all steps from gastro-intestinal interactions and absorption to systemic bio-

transformation, accumulation in body tissues and organs, and ultimate routes of excretion. The pharmacokinetic aspect of these steps must, of course, be taken into account. As discussed above, the Panel has considered knowledge of metabolic fate as falling into three general categories depending on whether it is known, can reasonably be assumed, or is unknown or uncertain.

Metabolic fate may be determined either by investigations conducted in intact living animals or by *in vitro* studies. The latter may range from experiments using digestive fluids simulating the gastro-intestinal secretions to more elaborate studies involving perfused organs, liver slices or homogenates, purified enzymes or intact cells or cell fractions derived from suitable tissues. A properly conducted *in vitro* study may stimulate what occurs *in vivo*, but there are many *in vivo* reactions for which there are no suitable *in vitro* models.

Many flavouring substances are highly volatile and are partially exhaled before and after reaching the digestive tract, where they may be absorbed unchanged. However, partial or complete breakdown into simpler chemical structures may occur within the intestinal tract by reaction with secretions, food components or microflora. The digestive fluids contain a wide variety of enzymes that function at acid pH in the stomach and at neutral or alkaline pH in the intestine, hydrolysing such compounds as esters, acetals, lactones and glycosides. Some of these enzymes may also act within the intestinal wall during absorption.

The process of transfer across the intestinal wall may be either active or passive. Simple sugars, fatty acids and amino acids are absorbed in part by active energy-dependent processes, and in part by passive diffusion. Small molecules, characteristic of most flavouring compounds and their metabolites, are generally absorbed by passive transfer. After absorption, compounds may undergo biochemical changes in the liver and, to a lesser extent, in other organs. The compounds and/or their metabolites are then excreted through the renal or intestinal pathways and, to a minor degree, by way of the lungs or sweat glands.

Most biotransformations are of the type that render foreign substances less toxic, hence the term 'detoxication'. This is definitely the case with flavouring compounds, the principal types of metabolic reactions being hydrolysis, oxidation, reduction and conjugation. The rate and extent to which the reactions occur vary with animal species, dietary composition, gastrointestinal contents and motility, and many other factors.

In almost all cases, a primary enzymatic route of biotransformation is largely or even exclusively followed at low doses. As this pathway becomes overloaded, e.g. as the capacity of these enzymes to deal with an absorbed substrate is exceeded, secondary systems may come into play or the substance may accumulate in some tissue and there induce an adverse reaction. Pathways of metabolism can be influenced by dose. Since large doses may overwhelm detoxication mechanisms, caution must be observed when making comparisons between the effects of very large doses and very small ones.

It should be observed that most metabolic studies, particularly the older ones performed before the advent of modern analytical methods, were conducted with doses sufficiently large to ensure recovery of either the unchanged substance or its metabolites. Such doses invariably were far in excess of the practical levels of use of flavour ingredients. The administration in metabolic studies of test doses sufficiently high to ensure the detection of metabolites by classical analytical methods, or the administration in feeding studies of levels sufficiently high to elicit adverse effects, may well involve atypical metabolic pathways or toxic consequences entirely different from those evoked under normal conditions of dietary intake of the test substances.

The Panel is fully aware of cases of metabolic conversion to compounds of increased toxicity, as occurs in the case of certain aromatic amines (e.g. 2-naphthylamine), chlorinated pesticides (e.g. heptachlor), and even safrole and estragole (Drinkwater, Miller, Miller & Pitot, 1976). The vast majority of flavouring substances, however neither exhibit this characteristic nor present a credible opportunity for it to occur at levels of human intake.

The metabolism of a compound of known structure can in most cases be inferred from known data on the metabolism of a related compound. However, suitable caution must be exercised on the basis of known exceptions and also because of the possibility of species differences. Where the metabolic fate is well established in several species of animals, and particularly in man, and the detoxifying or excretory capacity is sufficient to cope with any reasonably foreseeable intake, other kinds of information are useful primarily for confirmatory purposes.

The assurance with which one may predict metabolic fate would be limited indeed if such assumptions applied only to the high doses at which metabolic and toxicological studies are normally conducted. As pointed out above, the size of the dose of a substance can alter the pattern of its metabolism insofar as a large dose may exhaust the mechanism by which a small dose is metabolized. The compound may then accumulate temporarily, may be excreted partly in unchanged form or may undergo different metabolic reactions. Altered pathways are followed when certain conjugating agents (e.g. glycine and sulphate) are limited in supply but these pathways are not necessarily associated with increased toxicity.

The flavouring substances whose metabolic fate can reasonably be assumed, despite the paucity or absence of direct studies in animals, are those for which there is *in vitro* enzymatic evidence or a close chemical relationship to substances that at low intake levels are metabolized by the same routes and at essentially the same rates in man. Good examples are esters that are not sterically hindered and are hydrolysed readily by gastric, pancreatic, or hepatic esterases yielding moieties that are known to be safely metabolized. Most slightly branched aliphatic esters, many mononuclear aromatic aldehydes, phenols or acids, and simple alicyclic acids, esters, aldehydes and ketones fall into this group.

In the category of substances of unknown or uncertain metabolic fate, there is no reasonable biochemical basis for predicting toxicity or safety under condi-

tions of use. There may be structural variations contributing to possible steric hindrance by functional groups, leading to potentially hazardous metabolites. Classification here may reflect a lack of functional groups that would be expected to lead to ready metabolism or, in rare cases, a basic structure associated with more complex metabolism or significant toxicity.

Chemicals not normally present in food or markedly different from those found in food are regarded as xenobiotic. Examples are certain nitro compounds, aromatic amines, sulphones, isocyanates and a variety of heterocyclic compounds. In such cases, the metabolic fate may be unknown, or if it is suspected or known, it may suggest significant toxicity even at low dosage. Depending upon the potential use level, toxicological feeding studies, possibly of long duration, may need to be conducted. The Panel's policy has been, and continues to be, to withhold evaluation of any such potential flavouring substances until appropriate animal and/or metabolic studies have adequately demonstrated safety under the proposed conditions of use.

Toxicological protocols

As previously discussed, it is neither essential nor feasible to run toxicological studies in animals on every chemical in a structurally related group of flavouring substances. Far more useful is sufficient biochemical and metabolic information on prototypical members of a group, especially in a homologous series, to establish the relationship between chemical constitution and biological activity. In a stepwise or graduated series, quite valid predictions can be made from knowledge of the toxicity of other members of the series. The extensive toxicological investigations appropriate for the safety evaluation of pesticides, drugs and other biologically potent substances, residues of which may be present in foods as a result of their use in agriculture or animal husbandry, are rarely required in the case of the generally innocuous flavouring compounds, either natural or synthetic.

Setting these facts in proper perspective, the Panel has classified toxicological studies according to necessity and adequacy. Both are strongly affected by the available information on structure and metabolism. If substances belong to a well-studied and unexceptionally safe group carrying a strong initial presumption of safety, toxicological data on each specific substance are generally not needed. But if a substance is consumed at high levels and there is no previous experience with its use or it does not occur naturally in food, more direct toxicological data are essential. A less familiar structure for which the metabolic fate can reasonably be assumed would not require direct toxicity tests provided it belongs to a closely related group, members of which are without known toxic risk as shown by short-term toxicity tests. However, if the metabolic fate of such a substance is unknown, at least short-term tests on the specific substance are generally recommended, in addition to the requirement that it must belong to a group of closely related substances, several of which have been studied. Finally, if the structure is such that presumptions of safety are uncertain or negative, proper evaluation may pose the need for short-term tests and, in addition, long-term data on at least one member of the closely related group.

The details of toxicological test procedures need not be elaborated here. They are the subject of several reports emanating from national and international agencies and advisory committees (Boyland & Goulding, 1968; Food and Drug Administration, 1959 & 1970; Health Protection Branch, Canada, 1973; Joint FAO/WHO Expert Committee on Food Additives, 1958; Mehlman, Shapiro & Blumenthal, 1976; NAS–NRC, 1970 & 1975; Zbinden, 1973 & 1976). The protocols for acute, short-term and long-term oral feeding studies provide for observations in one or more laboratory species. Periodic observations are made of physical condition, behaviour, body weight, food efficiency, haematological and clinical responses, death rates and post-mortem pathology. In special cases, reproduction studies or tests for mutagenicity, teratology and carcinogenicity may be indicated.

The interpretation of toxicological tests requires the determination of a dosage level at which no adverse effects are observed. The limitations surrounding precise estimation of the no-adverse-effect level have been discussed in several papers and expert committee reports (Health Protection Branch, Canada, 1973; Joint FAO/WHO Expert Committee on Food Additives, 1958; NAS–NRC, 1975). This being a negative concept, it is imperative to understand and recognize adverse or toxic effects in experimental animals, such as failure to gain or maintain body weight, aberrations in physiological, clinical, anatomical or morphological parameters and abnormal reproduction.

In any case, the 'no-observed-adverse-effect' dose of the test substance, generally expressed in mg/kg body weight/day, is the critical dose which should be some large multiple of the potential daily human intake. The safety factor of 100 has been conventionally adopted for most food additives, but greater or lesser factors have frequently been used, for example by the Joint FAO/WHO Expert Committee on Food Additives, to arrive at acceptable daily intake (ADI) levels. Among the reasons for departing from the hundredfold safety factor are the similarity of a chemical substance to common food or body components, the nature and incidence of observed effects, demonstration of adverse effects at the lowest dosage tested, the slope of the dose-response curve and finally a long experience of safe use in food (Frazer, 1969).

Evaluation

Ultimately the process of safety evaluation of flavouring substances involves a balanced appraisal of all available information relevant to the criteria discussed above, whether or not the data are adequate either in amount or in kind. The FDA has acknowledged that: "To provide assurance that any substance is absolutely safe for human or animal consumption is impossible. This is particularly true for substances intended for human consumption which have been tested in animals" (21 CFR 121.3, 1976). However, reasonable conclusions concerning safety can be arrived at even where animal data are lacking. Many of the factors on which value judgements are based are not quantifiable and cannot be averaged or weighed against each other on an objective scale.

In evaluating the safety of environmental chemicals, it is recognized that "All decisions will necessarily be based on incomplete information. Accordingly, the most important element in the process of evaluating chemicals will be the mature judgment of experienced professionals" (NAS–NRC, 1975).

In instances where the data have not permitted an affirmative decision of safety under the specified conditions of use, the Panel has refrained from making such a judgement pending submission of further information, failing which it has rejected outright the proposed use of the material. Examples of the latter are methyl vinyl ketone and isoamyl isoeugenyl ether.

As previously discussed, lists of flavouring substances declared to be safe for their intended uses in major food classes have been published periodically in order to expose the Panel's judgements to scrutiny and possible criticism. The absence of sustainable challenge after a suitable, reasonably prolonged period following publication lends added support to GRAS status. In the event of a successful challenge, the substance in question is denied GRAS status. In a few instances, the Panel, like the FDA, has provided for interim use while additional toxicological information is being obtained.

Notwithstanding the advances in the past decade in identifying and reproducing the components of food flavours and in upgrading the criteria for safety evaluation, many hundreds of flavouring substances have been evaluated and determined to be safe under conditions of use. What is more, by far the majority of these judgements have withstood recent re-evaluation by the Panel. Where additional investigations may be required, it is because of appreciably higher intake levels, suspected carcinogenicity or new evidence of potential toxicity.

Toxicological tests of flavouring substances rate a low position on a priority scale compared with most food additives because of a combination of factors, notably their identity with or close similarity to the constituents of common foods, the minimal dietary intake levels, and the intrinsic sensory limitations on their use as additives to foods. The justification for such tests must be weighed against considerations of potential hazard under conditions of use, the importance of the substance in question and possible alternatives.

Safety evaluation demands constant vigilance. To be effective and in the best interests of both the food industry and the consuming public there should be continuous monitoring of the composition, use and consumption of flavouring substances, not only of those added in production but of changes in processing or home cooking, and changes in the pattern of food intake among various population groups. The role of scientists in both government and industry is one of great responsibility and, given the resources, will continue to be filled with high regard for the protection of public health.

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REFERENCES

Ambrose, A. M. & Sherwin, C. P. (1933). Detoxication mechanisms. *A. Rev. Biochem.* **2**, 377.

American Chemical Society (1969). Symposium on Natural Food Toxicants. *J. agric. Fd Chem.* **17**, 413.

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

Arctander, S. (1969). Perfume and Flavor Chemicals (*Aroma Chemicals*). Vols 1 and 2. S. Arctander, Montclair, New Jersey.

Bednarczyk, A. A., Galetto, W. G., Hall, R. L. & Stahl, W. H. (1975). New and improved flavor ingredients. In *Proceedings of IVth International Congress of Food Science and Technology.* p. 493.

Boyland, E. & Goulding, R. (Eds) (1968). *Modern Trends in Toxicology.* Vol. 1. Butterworths, London.

Central Institute for Nutrition and Food Research (1973). Lists of Volatile Compounds in Food. With supplements 1–7. dated 1975. TNO, Zeist, The Netherlands.

Drinkwater, N. R., Miller, E. C., Miller, J. A. & Pitot, H. C. (1976). Hepatorcarcinogenicity of estragole (1-allyl-4-methoxybenzene) and 1′-hydroxyestragole in the mouse and mutagenicity of 1′-acetoxyestragole in bacteria. *J. natn. Cancer Inst.* **57**, 1323.

Fenaroli's Handbook of Flavor Ingredients (1975). Edited by T. E. Furia and N. Bellanca. 2nd Ed. Vols I and II. CRC Press, Cleveland, Ohio.

Food and Drug Administration Advisory Committee on Protocols for Safety Evaluation (1970). Panel on Reproduction Report on Reproduction Studies in the Safety Evaluation of Food Additives and Pesticide Residues. *Toxic. appl. Pharmac.* **16**, 264.

Food and Drug Administration, Division of Pharmacology (1959). *Appraisal of the Safety of Chemicals in Foods. Drugs and Cosmetics.* The Association of Food and Drug Officials of the United States, Austin, Texas.

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

Food Protection Committee (1969). Guidelines for Estimating Toxicologically Insignificant Levels of Chemicals in Food. National Academy of Sciences–National Research Council, Washington, D.C. (Reprinted as an Appendix to report of National Academy of Sciences–National Research Council, 1970).

Food Protection Committee (1972). A Comprehensive Survey of Industry on the Use of Food Chemicals Generally Recognized As Safe. National Academy of Sciences–National Research Council, Washington, D.C.

Food Protection Committee (1973). *Toxicants Occurring Naturally in Foods.* 2nd Ed. National Academy of Sciences–National Research Council. Washington, D.C.

Frazer, A. C. (1969). Health aspects of artificial sweeteners. *J. R. Soc. Health,* **89**, 133.

Gerarde, H. W. (1973). Survey update: Determining safety for flavor chemicals. *Fd Prod. Dev.* **7**, 82.

Gontzea, I. & Sutzescu, P. (1968). *Natural Antinutritive Substances in Foodstuffs and Forages.* S. Karger, Basel.

Guenther, E. (1948–52). *The Essential Oils.* Vols I–VI. D. Van Nostrand, Inc., Princeton, New Jersey.

Hall, R. L. (1976). Estimating distribution of daily intakes of certain GRAS substances. In *Committee on GRAS List Survey—Phase III.* National Academy of Sciences–National Research Council, Washington, D.C.

Hall, R. L. & Oser, B. L. (1961). Recent progress in the consideration of flavoring ingredients under the Food Additives Amendment. II. *Fd Technol., Champaign* **15** (12), 20.

Hall, R. L. & Oser, B. L. (1965). Recent progress in the consideration of flavoring ingredients under the Food Additives Amendment. III. GRAS substances. *Fd Technol., Champaign* **19** (2), part 2, 151.

Hall, R. L. & Oser, B. L. (1968). The safety of flavoring substances. *Residue Reviews* **24**, 1.

Hall, R. L. & Oser, B. L. (1970). Recent progress in the consideration of flavoring ingredients under the Food Additives Amendment. 4. GRAS substances. *Fd Technol., Champaign* **24** (5), 25.

Handler, P. & Perlzweig, W. A. (1945). Detoxication mechanisms. *A. Rev. Biochem.* **14**, 617.

Hathway, D. E. (1970, 1972 & 1975). *Foreign Compound Metabolism in Mammals.* Vols 1–3. The Chemical Society, London.

Health Protection Branch, Canada (1973). The Testing of Chemicals for Carcinogenicity-Mutagenicity-Teratogenicity. Ministry of National Health and Welfare, Ottawa.

Joint FAO/WHO Expert Committee on Food Additives-Second Report (1958). Procedures for the Testing of Intentional Food Additives to Establish Their Safety for Use. Tech. Rep. Ser. Wld Hlth Org. **144.**

Liener, I. E. (Ed.) (1969). *Toxic Constituents of Plant Foodstuffs.* Food Science and Technology: A Series of Monographs. *Academic Press, New York.*

Mehlman, M. A., Shapiro, R. E. & Blumenthal, H. (Eds) (1976). *Advances in Modern Toxicology. Vol I, part 1: New Concepts in Safety Evaluation.* John Wiley & Sons, New York.

National Academy of Sciences–National Research Council (1970). Evaluating the Safety of Food Chemicals. NAS–NRC, Washington, D.C.

National Academy of Sciences–National Research Council (1975). Principles for Evaluating Chemicals in the Environment. NAS–NRC, Washington, D.C.

Oser, B. L. (1969). Much ado about safety. *Fd Cosmet. Toxicol.* **7**, 415.

Oser, B. L. (1970). A comparison of the safety-in-use of natural and synthetic flavors. Proceedings of IIIrd International Congress of Food Science and Technology. p. 771.

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

Oser, B. L. & Ford, R. A. (1973a). Recent progress in the consideration of flavoring ingredients under the Food Additives Amendment. 6. GRAS substances. *Fd Technol., Champaign* **27** (1), 64.

Oser, B. L. & Ford, R. A. (1973b). Recent progress in the consideration of flavoring ingredients under the Food Additives Amendment. 7. GRAS substances. *Fd Technol., Champaign* **27** (11), 56.

Oser, B. L. & Ford, R. A. (1974). Recent progress in the consideration of flavoring ingredients under the Food Additives Amendment. 8. GRAS substances. *Fd Technol., Champaign* **28** (9), 76.

Oser, B. L. & Ford, R. A. (1975). Recent progress in the consideration of flavoring ingredients under the Food Additives Amendment. 9. GRAS substances. *Fd Technol., Champaign* **29** (8), 70.

Oser, B. L. & Ford, R. A. (1977). Recent progress in the consideration of flavoring ingredients under the Food Additives Amendment. 10. GRAS substances. *Fd Technol., Champaign* **31** (1), 65.

Oser, B. L. & Hall, R. L. (1972). Recent progress in the consideration of flavoring ingredients under the Food Additives Amendment. 5. GRAS substances. *Fd Technol., Champaign* **26** (5), 35.

Parke, D. V. (1968). *The Biochemistry of Foreign Compounds.* International Series of Monographs in Pure and Applied Biology, Biochemistry Division. Vol. 5. Pergamon Press, Oxford.

Roe, F. J. C. (Ed.) (1970). *Metabolic Aspects of Food Safety.* Blackwell Scientific Publications, Oxford.

Sherwin, C. P. (1922). The fate of foreign organic compounds in the animal body. *Physiol. Rev.* **2**, 238.

Stekol, J. (1941). Detoxication mechanisms. *A. Rev. Biochem.* **10**, 265.

United States Dispensatory (1967). 26th Ed. Edited by A. Osol, R. Pratt and M. D. Altschule. J. B. Lippincott Company, Philadelphia.

WHO Scientific Group (1967). Procedures for Investigating Intentional and Unintentional Food Additives. *Tech. Rep. Ser. Wld Hlth Org.* **348.**

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

Zbinden, G. (1973 & 1976). *Progress in Toxicology. Special Topics.* Vols 1 & 2. Springer-Verlag, New York.

Fd Cosmet. Toxicol. Vol. 15. p. 467. Pergamon Press 1977. Printed in Great Britain

REVIEWS OF RECENT PUBLICATIONS

Environmental Mercury and Man: A Report of an Inter-Departmental Working Group on Heavy Metals. Pollution Paper No. 10. Central Unit on Environmental Pollution. Department of the Environment. HMSO. London. 1976. pp. viii + 92. £1.40.

In common with its predecessor on lead (Department of the Environment. Pollution Paper No. 2. HMSO. London. 1974; pp. viii + 47. £0.45) this report on mercury reviews sources and levels in the environment, pathways to man via food, drink, air and skin absorption, fate in the body, the exposure of the general public and of industrial workers, and the types of poisoning associated with different classes of mercury compounds. Other topics discussed are controls on levels in food, drink, consumer goods and pesticides, on emissions to air and water and on disposal of wastes, and facilities for monitoring and research are outlined. Appendices provide more details of sources and environmental concentrations, including tables of levels detected in air, water and soil.

It is concluded that for most people food is by far the most significant source of mercury exposure, and official UK surveys (*Cited in F.C.T.* 1974, **12**, 139) have indicated that average intakes are well within provisional tolerable intakes set by FAO/WHO. Nevertheless, there is a need for special consideration in cases of high exposure resulting from unusual diets or high atmospheric levels, or in medical disorders that may produce a low tolerance to mercury. Further information is regarded as necessary on levels in air and water, monitoring for which has already been initiated, and on conversion between different forms of mercury and pathways through the environment. Those working in dental practices are singled out for regular examination for excessive mercury absorption, and the report suggests that it may be desirable to extend this examination to their families and those of other mercury workers. More research is also required on the mechanisms of toxic action of mercury, and on the effects of low-level exposure (particularly to organic compounds) over long periods of time.

In general it is recommended that mercury intakes should be kept as low as possible. Although precipitate action is unnecessary, man-made contamination should be progressively reduced as and when appropriate, taking into account industrial viability, technical adequacy and the availability of less hazardous substitutes. It is noted that in the chloralkali industry, progress has been made in reducing mercury losses to the environment, and the diaphragm process, which uses no mercury, is gradually being adopted. The need for continued use of mercury in paints is also under review. Research into a substitute seed treatment is continuing, and the Advisory Committee on Pesticides and Other Toxic Chemicals has in hand a detailed review of the use of mercury in agricultural pesticides.

Fd Cosmet. Toxicol. Vol. 15. pp. 469-473. Pergamon Press 1977. Printed in Great Britain

BOOK REVIEWS

Carbamate Insecticides: Chemistry, Biochemistry, and Toxicology. By R. J. Kuhr and H. W. Dorough. CRC Press, Inc., Cleveland, Ohio, 1976. pp. vi + 301. $63.50.

Carbamates are a comparatively recent addition to the important group of agricultural chemicals used to control insect pests. The first compound was marketed in the mid-1960s. Since then a number of others have appeared on the scene and this CRC manual on carbamate insecticides is a timely reminder that this group of chemicals may acquire considerable importance in the future, as the older persistent pesticides, particularly the organochlorines, are phased out of commercial use.

The origin of this group of compounds can be traced to the Calabar bean, the juice of which contains the lethal alkaloid eserine or physostigmine, a carbamate with pronounced cholinergic effects that was used extensively at one time on the West African coast of Calabar in a form of trial by ordeal. The discovery that other members of the group possessed the same type of pharmacological activity and were lethal to insects as well as to mammalian species led to a probing search for compounds that were selectively toxic to insects. Out of several hundreds of compounds synthesized, a few have found their way into commercial use. All are potent insecticides, but they are also toxic to mammals at relatively high concentrations.

The book deals systematically with the structure of some key carbamate insecticides, particularly those that are on the market, and with their toxic potency to target and non-target organisms. It also deals extensively with metabolism in mammals and with breakdown of the compounds in the environment. The mechanisms by which the toxic effects are exerted in both insects and mammalian species are also treated in some depth.

The section on toxicology is, unfortunately, not as comprehensive as one might expect. Comparative data on acute toxicity, especially LD_{50} values, are given, but little attempt is made to evaluate the data critically and to relate this to experience in man. In particular, no effort is made to relate the depression of serum-cholinesterase activity, a widely used index of exposure to carbamates, with biological signs of toxicity. There is very little mention of subacute or long-term work and no mention at all of any work on the pathological changes in the central nervous system of carbamate-exposed animals. Perhaps information of this sort is inadequate or does not exist, but the authors should have made a more positive identification of these gaps in order to guide future research.

Apart from this, however, toxicological aspects are well covered. The authors seem well aware of and give adequate attention to the environmental impact of this new class of pesticide, particularly in relation to questions of persistence and toxicity to non-target species. They also provide thorough coverage of the problem of tissue residues in mammals and particularly in man.

The book is readable and well written and the reader should find little difficulty in following the account of the development, uses and biological and environmental effects of carbamates. It should be on the shelf of every toxicologist, since there is a good chance that this group of compounds will assume much greater economic importance in the future.

Environmental Quality and Safety. Global Aspects of Chemistry, Toxicology and Technology as Applied to the Environment. Vol. 4. Edited by F. Coulston and F. Korte. Georg Thieme Verlag, Stuttgart; Academic Press, New York, 1975, pp. viii + 276. £10.15.

This volume is slanted towards an understanding of the hazards involved in environmental contamination, and the contributions include a number based on papers presented at various international symposia and other meetings concerned with ecology and the quality of the environment. Several authors have contributed short articles—one could almost call them essays—on insecticides and other pesticides, with particular emphasis on the persistence of these products in various types of soil and on the ways in which they are degraded. Attention is directed mainly to the principal groups of chemicals used in this connexion, namely the organochlorine, organophosphorus and carbamate insecticides, but the less widely used organotin compounds are discussed in an interesting article by D. D. McCollister and A. E. Schober, and several contributions concentrate on herbicides, including paraquat.

Problems of environmental pollution of a less specific nature are also considered. Two articles relate mainly to the identification of noxious chemicals in the atmosphere and one of these gives details of concentrations and the amounts likely to be respired by man. An account is also given of various types of manufactured chemicals that are likely to find their way into the environment either from the production site or during or after use.

The articles are fairly general in approach and lack the authority of in-depth reviews. They are, however, extremely useful for newcomers to toxicology and for the experienced but busy toxicologist who requires a reasonably short introduction to a topic with which he is unfamiliar. The book thus fills an important gap between original articles and authoritative reviews. It is somewhat surprising that the dangers of radiation in the environment are treated in only one brief article. Surely the public concern about this topic should have prompted the editors to look more closely into this problem. Lack of space is unlikely to have been the reason for this neglect, since the

book includes two papers on the methods used for identification of mutagens and carcinogens. These, while of general interest to the toxicologist, do not seem to fit in with the rest of the contributions, with their accent on the occurrence and fate of environmental contaminants.

Anticonvulsant Drugs and Enzyme Induction. Study Group 9 of the Institute for Research into Mental and Multiple Handicap. Edited by A. Richens and F. P. Woodford. Associated Scientific Publishers, Amsterdam, 1976. pp. viii + 203. Dfl. 46.50.

It is well known that the microsomal fraction of mammalian liver contains an enzyme complex capable of metabolizing a variety of endogenous substrates, such as steroids and fatty acids, and a multitude of exogenous substances (xenobiotics), including drugs, pesticides, food additives, carcinogens and other environmental toxins. Studies using experimental animals have shown that the activities of hepatic microsomal xenobiotic-metabolizing enzymes may be increased by treatment with various compounds including phenobarbitone, phenytoin, ethosuximide and certain other anticonvulsant drugs used in the treatment of epilepsy.

The monograph under review consists of seventeen research papers presented at a symposium organized by the Institute for Research into Mental and Multiple Handicap and held in London at the end of April 1975. Each paper is supplemented by a reference list and a synopsis of the discussion that followed its presentation at the meeting. The final symposium discussion, on tests for hepatic microsomal-enzyme induction in man, the diurnal variation of drug metabolism and the possible therapeutic uses of enzyme induction, is also included. A subject index for the whole volume is provided.

A number of papers are concerned with the detection of hepatic microsomal-enzyme induction in man. Although many studies on enzyme induction have been conducted in experimental animals, comparatively few investigations have been performed in man. In animal experiments hepatic enzyme levels may readily be assessed by direct determinations on liver tissue, whereas in man these estimations, unless conducted on small needle-biopsy samples, must be obtained by indirect indices, including the determination of the half-life of drugs such as antipyrine, the measurement of serum γ-glutamyl transpeptidase and the urinary excretion of D-glucaric acid and 6β-hydroxycortisol. From the data presented by various authors it appears that no single test can be relied upon, at present, as an index of human enzyme induction. This problem is highlighted by the studies of E. S. Vesell, who reports that the administration of phenobarbitone to normal subjects produces a marked variation in the induction of antipyrine metabolism as assessed by half-life measurements. However, studies with both identical and fraternal twins reveal that the large inter-individual variations observed in man are due to genetic rather than environmental factors.

The long-term administration of anticonvulsant drugs to both man and experimental animals is known to lead to a number of side-effects which may be attributable to the induction of hepatic microsomal enzymes. In this monograph several papers are presented on the disturbance of both vitamin D and folic acid metabolism in epileptics and on the incidence of certain clinical conditions such as hypocalcaemia, osteomalacia, gingival hyperplasia and tooth-root abnormalities. Furthermore, whilst anticonvulsant drugs induce their own metabolism, they may also accelerate the formation of therapeutically ineffective metabolites of other drugs administered to epileptics.

A paper presented by M. G. Horning and his co-workers deals with the detection and quantification of anticonvulsant drugs and their metabolites in biological fluids by combined gas chromatography–mass spectrometry. Finally, papers are included on the use of isolated hepatocytes in *in vitro* studies on drug metabolism and on the isolation and purification of components of the hepatic microsomal xenobiotic-metabolizing complex.

This book is of great importance to anyone concerned either with the clinical effects of anticonvulsant-drug administration or with the detection and measurement of microsomal-enzyme induction in man.

Immunological Aspects of Neoplasia. A Collection of Papers Presented at the Twenty-Sixth Annual Symposium on Fundamental Cancer Research, 1973. Published for The University of Texas System Cancer Center, M. D. Anderson Hospital and Tumor Institute, Houston, Texas. The Williams & Wilkins Company, Baltimore, Md, 1975. pp. xiii + 733. $30.00.

This collection of papers presented at the Twenty-Sixth Annual Symposium on Fundamental Cancer Research, held in Houston, Texas, in 1973, covers practically every aspect of experimental tumour immunology. The papers are grouped into sections, which deal with recent advances in basic immunology, cell-surface antigens in neoplasia, immunological changes in neoplasia, and immunotherapy, the two latter topics each being considered in two separate parts. Each section begins with an introductory chapter written by a recognized authority in the field and continues with a presentation of basic work supported by numerous references.

This is a book for the specialist but not necessarily the immunological specialist. It could be recommended for the experimentalist in the cancer field, but a proper understanding of the contents would require some familiarity with immunological techniques. The sections on immunotherapy will obviously have an appeal for clinicians. It is interesting, however, for investigators who work mainly with model systems to see how the fruits of their research are being applied to man, and for this group these chapters should provide welcome background information.

For most people the usefulness of a book of this type is twofold. First it shows the direction in which tumour immunology is progressing and secondly it provides considerable insight into current experimental approaches. The problem with this particular

book stems from the fact that the congress was held in 1973, and at the current rate of immunological progress it seems reasonable to ask how much of its content is still relevant only a year or so after publication.

An Introduction to Epidemiology. By M. Alderson. Macmillan Press Ltd., London, 1976. pp. x + 226. £4.50.

The success of epidemiology in the search for hazards to health in the past 20 years has brought the subject to the attention of many who had previously thought of it as an activity confined to dusty offices strewn with paper.

This volume is the outcome of the author's experience of teaching the subject, but its usefulness extends beyond those for whom epidemiology may become a career. For instance, the general practitioner often has data to which epidemiological techniques may be applied; W. N. Pickles, working in rural Yorkshire nearly 40 years ago used simple methods to provide "unique contributions on the spread of a number of infectious diseases". Others for whom some knowledge of epidemiology is useful are laboratory workers, whose findings may identify a need for epidemiological work. For those working in public health or toxicological laboratories, this volume identifies the methods and the difficulties of relating to human populations data derived from animal experimentation.

Many will dispute the author's claim that epidemiology can, in the strictest sense, identify the aetiological agents of disease. However, the techniques and results of the epidemiologist, considered in conjunction with laboratory findings, may establish causal factors with a degree of certainty that warrants attention and action by government or those working in health services.

The restriction of the text to some 200 pages and the inclusion of further reading lists and references, an adequate index and many examples all combine to make this volume a desirable acquisition for those interested in the causes of disease.

The Cell in Medical Science. Vol. 4. Cellular Control Mechanisms: Cellular Responses to Environment. Edited by F. Beck and J. B. Lloyd. Academic Press Inc., (London) Ltd. pp. xiv + 429. £11.80.

Considerable strides have been made in the past decade in the elucidation of the mechanisms by which cells maintain their integrity and function as units of a complex multicellular organism. Unfortunately these advances in one of the basic biological sciences have had little impact on some important areas of applied biology, notably medicine and toxicology. The reason for this is not difficult to find. The basic biological sciences, and that of cell biology in particular, have generated a new vocabulary of terms and a new philosophy of approach, which can generally be assimilated only in the course of several years of relevant work, so it is very hard to transfer this new knowledge into the crowded curricula of the applied sciences.

It appears, however, that many of the major points of controversy in this field have now become sufficiently clear for writers and teachers to give a concise and connected account of important cell processes. This is reflected in the book named above, which presents a group of contributions that are both comprehensive and readable. They are almost free of jargon and emphasize the areas of general agreement; while controversial issues are not totally ignored their discussion is not allowed to distract the reader from the main theme. Although different authors are responsible for each chapter, a uniform approach is maintained, each topic being discussed from the point of view of cellular control mechanisms.

The first two chapters deal with the intracellular control of enzyme activity and of protein synthesis, while the third forms a useful link between intracellular and intercellular control processes, dealing with the part of cell activity that is designed to influence adjacent or distant cells. The rest of the book is devoted to the response of the cell to extracellular influences, generally the products of other cells. Among this group of contributions, the chapter on immunology deserves particular commendation. Although it may appear superficial to the expert immunologist, it contains a most useful summary of up-to-date knowledge for the interested non-specialist. The chapters on acute inflammation, wound healing and cancer and carcinogenesis are less informative, no doubt because the subjects are vast and the space allotted seems too small to do them adequate justice. Perhaps it would have been more satisfactory to omit altogether the chapter on the macrophage and allocate the extra space to these three important topics. Indeed it is difficult to see why the information on the macrophage was not included in the chapter on immunology.

This book should be of great interest to those biologists who are keen to keep up with the main areas of advance in the basic biological sciences in the expectation of finding information that may be put to good use in the applied field.

Laboratory Techniques in Biochemistry and Molecular Biology. Techniques of Sample Preparation for Liquid Scintillation Counting. By B. W. Fox. North Holland Publishing Company, Amsterdam, 1976. pp. 333. $18.50.

This book, which is a soft-cover edition of volume 5 part I of the series *Laboratory Techniques in Biochemistry and Molecular Biology*, is clearly aimed at the laboratory worker involved in the measurement of radioactivity from weak beta-emitting isotopes, which are being used increasingly in many areas of investigation, particularly biochemistry and environmental studies. The use of the correct sample-preparation technique is crucial in work with radioisotopes, and an up-to-date survey of this topic is therefore particularly valuable. The extensive literature in this field, last reviewed by J. C. Turner in a Radiochemical Centre Monograph in 1971 (in considerably less detail than in the present volume) is spread through a wide

range of books and journals not always readily available to the interested laboratory worker.

The first chapter in the book is a brief introduction to the general principles of liquid scintillation spectrometry, dealing with the physics of the scintillation phenomenon, the various forms of quenching experienced and some practical points on scintillation vials and instrumentation. This is followed by a chapter covering homogeneous and heterogeneous counting systems and systems capable of utilizing Cerenkov radiation. Included in this chapter is a section on colloid counting and the construction of phase diagrams.

Following a chapter on the general aims of preprocessing techniques, specific aspects of sample preparation are considered in relation to animal tissues, including blood, urine and faeces, to plant tissues and soil samples, to cells in culture and to isolated macromolecules. The many problems encountered in preparing tissue extracts and chromatographic eluates for counting are covered in a separate chapter, as is the preparation of electrophoretic and chromatographic support media. A section on centrifugation seems rather out of place in this chapter and would fit more naturally into the previous chapter on macromolecules. The remainder of the book covers applications of the technique to inorganic materials, geophysics and archaeology and a few miscellaneous topics, and includes a lengthy and valuable section on quench-correction techniques, multiple isotope counting and data handling.

Finally the book has a number of useful appendices, providing isotope tables and decay charts, properties of solutes, solvents and scintillation mixtures, and data on spectrometer manufacturers and commercially available scintillation cocktails. A reference section cites about 500 publications from the literature up to 1973. In all, this is a useful, well written and well laid-out book, which should be of considerable value to the research worker using radioisotopes at the bench.

Progress in Medicinal Chemistry. Vol. 13. Edited by G. P. Ellis and G. B. West. North-Holland Publishing Company, Amsterdam, 1976. pp. x + 357. $43.95.

This volume, the thirteenth in a series devoted to specialist topics in the field of biologically related subjects contains five review articles. In the first, which is by far the most extensive, D. M. Goldberg deals with general, technical and biological aspects of several enzymes of considerable importance in the diagnosis of clinical conditions in man. Additionally, he provides a comprehensive and critical assessment of the value of diagnostic enzymology in genetic disorders and in prenatal diseased states. This is an excellent review of an extremely complex and important subject and will undoubtedly be of value to clinical chemists.

The remaining reviews are more esoteric. The second, by P. F. L. Boreham and I. G. Wright, is concerned with pathological processes in parasitic diseases, relating these to the release of pharmacologically active compounds. The substances reviewed include the kinin system, histamine and 5-hydroxytryptamine, catecholamines and the prostaglandins. The information available on these topics is scattered throughout many scientific journals and the authors have undertaken the difficult task of collating this information and drawing what limited conclusions are possible. Clearly, this increasingly important aspect of parasitology merits further attention.

The third review deals with the 1,2,3-triazines, a class of compound of potential importance in medical applications. In the absence of definitive evidence that these compounds interact covalently with macromolecules, which would provide a sound scientific basis for their biological reactivity, M. F. G. Stevens confines his attention largely to the chemistry of the substituted 1,2,3-triazines.

The fourth review, by A. D. Russell and D. Hopwood, is on the chemistry of glutaraldehyde and its biological applications, particularly in the fixation of tissues for electron microscopy, and finally G. Doyle Daves and C. C. Cheng discuss the chemistry and biochemistry of *C*-nucleosides. In contrast to the *N*-nucleosides, these compounds are distinguished by the hydrolytic stability of the carbon–carbon bond forming the ribosidic linkage, and have recently come into prominence following their discovery in a number of antibiotics. These *C*-nucleoside antibiotics exhibit diverse biological effects, including potentially useful antitumour and antiviral activities.

The contributions in this volume maintain the high standard associated with the earlier members of the series and will doubtless be of value to specialists interested in the particular topics covered.

BOOKS RECEIVED FOR REVIEW

Histological Techniques. By M. Gabe. Springer-Verlag, Berlin, 1976. pp. xxiii + 1106. DM 120.80.

Environmental Pollution and Carcinogenic Risks: Proceedings of a Symposium organized by IARC and the French National Institute of Health and Medical Research, held in Lyon, France, 3–5 November 1975. Edited by C. Rosenfeld and W. Davis. IARC Scientific Publications no. 13. Agency for Research on Cancer, Lyon, 1976. pp. 454. Sw.fr. 50.00.

Environmental *N*-Nitroso Compounds—Analysis and Formation. Proceedings of a Working Conference held at the Polytechnical Institute, Tallinn, Estonian SSR, 1–3 October 1975. Edited by E. A. Walker, P. Bogovski and L. Griciute. IARC Scientific Publications no. 14. International Agency for Research on Cancer, Lyon, 1976. pp. iii + 512. Sw.fr. 110.00.

IARC Monographs on the Evaluation of Carcinogenic Risk of Chemicals to Man: Some Carbamates, Thiocarbamates and Carbazides. Vol. 12. International Agency for Research on Cancer, Lyon, 1976. pp. 282. Sw.fr. 34.00.

Survival Distributions: Reliability Applications in the Biomedical Sciences. By A. J. Gross and V. A. Clark. John Wiley & Sons Ltd. Chichester, 1976. pp. xv + 331. £14.60.

Biochemical Toxicology of Environmental Agents. By A. De Bruin. Elsevier Scientific Publishing Company, Amsterdam, 1976. pp. x + 1544. Dfl. 320.00.

The Prediction of Chronic Toxicity from Short Term Studies. Proceedings of the Meeting held at Montpellier, June 1975. Proceedings of the European

Society of Toxicology. Vol. 17. Edited by W. A. M. Duncan, B. J. Leonard and M. Brunaud. Excerpta Medica, Amsterdam, 1976. pp. viii + 440. $61.50.

Side Effects of Drugs Annual I 1977. A Worldwide Yearly Survey of New Data and Trends. Edited by M. N. G. Dukes. Excerpta Medica, Amsterdam, 1977. pp. xvii + 420. $38.50.

Fortschritte in der Lebensmittelwissenschaft. Nr. 4. Amine und Nitrosamine: Vorkommen, Bedeutung, Stoffwechsel und Bestimmung. By A. Askar. Technische Universität Berlin. Berlin, 1976. pp. vi + 221. DM 10.50.

Glutathione: Metabolism and Function. Kroc Foundation Series, Vol. 6. Edited by I. M. Arias and W. B. Jakoby. Raven Press. New York, 1976. pp. xiii + 382. $28.50.

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

Immunology of the Gut. CIBA Foundation Symposium 46. Edited by R. Porter and J. Knight. Excerpta Medica, Amsterdam, 1977. pp. viii + 376. $28.00.

Handbook of Industrial Toxicology. By E. R. Plunkett. Heyden & Son Ltd., London, 1976. pp. 552. £17.50.

The Genetics and Biology of Drosophila. Vol. 1b. Edited by M. Ashburner and E. Novitski. Academic Press Inc., (London) Ltd., London, 1976. pp. li + 468. £16.80.

Residue Reviews: Residues of Pesticides and Other Contaminants in the Total Environment. Vol. 63. Edited by F. A. Gunther. Springer-Verlag, New York, 1976. pp. viii + 193. DM 41.00.

Residue Reviews: Residues of Pesticides and Other Contaminants in the Total Environment. Vol. 64. Edited by F. A. Gunther. Springer-Verlag. New York, 1976. pp. ix + 142. DM 41.00.

Current Approaches in Toxicology. Edited by B. Ballantyne. John Wright & Sons Ltd., Bristol. 1977. pp. viii + 310. £8.50.

Datensammlung zur Toxikologie der Herbizide. Deutsche Forschungsgemeinschaft-Kommission für Pflanzenschutz-, Pflanzenbehandlungs- und Vorratsschutzmittel. Series 2. Verlag Chemie GmbH, Weinheim, 1976. pp. 176. DM 98.

Mammalian Cell Membranes. Vol. 1. General Concepts. Edited by G. A. Jamieson and D. M. Robinson. Butterworths, London, 1976. pp. ix + 276. £18.00.

Mammalian Cell Membranes. Vol. 2. The Diversity of Membranes. Edited by G. A. Jamieson and D. M. Robinson. Butterworths, London, 1976. pp. ix + 364. £18.00.

Mammalian Cell Membranes. Vol. 3. Surface Membranes of Specific Cell Types. Edited by G. A. Jamieson and D. M. Robinson. Butterworths, London, 1976. pp. ix + 276. £18.00.

Fd Cosmet. Toxicol. Vol. 15, pp. 475–485. Pergamon Press 1977. Printed in Great Britain

Information Section

ARTICLES OF GENERAL INTEREST

MULTIPLE ENVIRONMENTAL CARCINOGENS: THEIR SIGNIFICANCE*

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The term 'cancer' covers a variety of clinical states differing considerably in their clinical appearance, prognosis and growth behaviour, and not least in their aetiology As it is impossible to consider all the many causes of malignant tumours in a review article, I shall restrict my comments to the field of chemical carcinogenesis, considered by many experts to be one of the most important factors in the genesis of malignant tumours. It is assumed today that more than 80% of all human tumours can be attributed to the action of exogenous chemical factors.

First I should like to mention briefly some long-standing and well-known examples of chemical carcinogenesis, which have been discussed in some detail elsewhere (Schmähl, *Entstehung, Wachstum und Chemotherapie maligner Tumoren*, 2nd ed.; Editio Cantor, Aulendorf, 1970). These include the carcinogenic action of soot and tar on human skin and of some inorganic substances (such as chromium, nickel and beryllium) on the upper respiratory tract, the induction of bladder cancer by occupational exposure to several aromatic amines and the more or less specific induction of mesotheliomas by asbestos. These effects have been detected mainly in studies of so-called occupational carcinogenesis, and new factors leading to occupational cancers continue to be demonstrated; only recently vinyl chloride has been associated with a relatively specific induction of haemangiosarcomas, mainly of the liver (IARC International Technical Report no. 75/001, Lyon, 1975) and various haloethers have been shown to induce lung cancer (Figueroa *et al. New England. J. Med.* 1973, **288,** 1096).

In the past two decades, conclusive indications of causes of other human tumours have been derived from studies of the occurrence of specific types of tumour in various geographical areas (Doll *et al. Cancer Incidence in Five Continents*; Springer Verlag, Berlin, 1970), a line of enquiry that has yielded very interesting and exciting observations. Thus among the population of Japan, the incidence of gastric cancer is five times as high as in the white population of the United States, but even within Japan the incidence in different areas may vary by a factor of two. A slight-to-moderate regression of stomach cancer is being noted in all parts of the world except Japan, where this type of cancer is actually increasing. Racial factors do not seem to be involved, since Japanese emigrants to the United States show, after a fairly short time, the same risk of cancer of the digestive tract as the American population, namely a relatively low rate of stomach cancer and a high rate of cancer of the colon and rectum. The factors responsible for the development of stomach cancer are considered to act on the affected individuals during their youth (Haenszel & Correa, *Cancer Res.* 1975, **35,** 3545).

The increase in cancer of the bronchi is another problem altogether. This type of cancer occurs about four times as frequently in Great Britain as in Japan, and there must be specific reasons for this, too. Today it is generally considered that inhalation of tobacco smoke and air polluted with carcinogenic substances is the main cause of this type of cancer (Schmähl, *Dtsch. med. Wschr.* 1975, **100,** 965), which thus represents a prototype of chemically induced cancer.

In Assam, 57% of all cancers are sited in the digestive organs, while in Bulgaria, skin cancer in farmers accounts for 72% of all cases of cancer, probably because the Bulgarian rural population is exposed to sunlight to a particularly high degree. The incidence of cancer of the mouth varies widely in different parts of the world, occurring some 35 times more frequently in India, Ceylon and Thailand than in Europe and accounting for 30% of all types of cancer in Asia but for only 0·5–1% in Europe. It is now known that the chewing of betel nuts together with raw tobacco leaves causes this type of cancer. Another wide variation is seen within Africa in the relative incidence of different types of uterine cancer. In this case, sexual hygiene is the exogenous factor implicated, and the ratios of corpus to cervical carcinoma have been found to range from 1:0·1 in Egypt to 1:4·0 in the Sudan, 1:15·2 in Mozambique and 1:44·7 in South Africa (Huber, *Medsche Klin.* 1968, **63,** 1136). The corresponding ratio for Europe is 1:4·0–6·0.

Finally, a particularly instructive example was discovered relatively recently (Kment & Mahboubi, *Science, N.Y.* 1972, **175,** 846) in specific regions on the Caspian Sea. Here the incidence of oesophageal cancer is 30 times higher than in other parts of Persia or Europe and women are more frequently afflicted than men, although the reverse is usually the case with this type of tumour. It is clear that there must be a specific cause for this high incidence and in Heidelberg we are studying the diets of the populations living in the Caspian Sea area in the hope of identifying constituents known to induce cancer of the oesophagus experimentally.

*Based on the text of an address to the BIBRA Annual Scientific Meeting held in London on 6 December 1976.

More examples could be quoted, but those mentioned may show that cancer is due in many cases to environmental factors and often reflects customs and habits. The oldest example of this is cancer of the penis, which hardly ever occurs in Jews, who are circumcised shortly after birth, but is often found in men whose sexual hygiene is inadequate, the incidence ratio in these two groups being something like 1:300. Thus postnatal circumcision is, historically, the first example of cancer prophylaxis—unrecognized, but nevertheless extremely effective—and furthermore, it is clear that protection against a specific type of cancer can be achieved simply by the frequent use of soap and water (for the removal of smegma).

Several problems are given priority in the work of our institute in Heidelberg because they seem to be of great topical interest in relation to purposeful research into chemical carcinogenesis. From the toxicological viewpoint, chemical carcinogens can be divided into at least two groups, namely those that have local effects and those that have systemic effects. The former lead to cancer at the site of application, while the latter induce tumours in specific organs or even in several parts of the body (multipotent carcinogens). Whereas bronchial cancer, for example, is generally induced by locally acting carcinogens, which reach their site of action by direct contact of contaminated inspired air with cells of the bronchial mucosa, most of the occupational bladder cancers are induced by absorbed carcinogens of the aromatic amine type. A clear distinction between local and systemic carcinogens is not always possible in practice, but the division has proved useful, especially when expert judgements are concerned.

Now I should like to deal briefly with diaplacental carcinogenesis, a subject that has been investigated seriously only during the last 10 years but is of considerable practical significance (Ivankovic, *Handbuch allg. Path.* 1975, **6,** 942). Various chemical compounds (e.g. several nitrosamides, procarbazine and certain polycyclic aromatic hydrocarbons) may act directly on the foetus, inducing germ layers of tumour cells which only manifest themselves later, in postnatal life. If, for example, rats or other small rodents are given a single oral or intravenous dose of ethyl nitrosourea, equivalent to only 0·5–5·0% of the LD_{50}, the offspring appear healthy at birth but more than 90% develop malignant tumours of the brain and central and peripheral nervous systems after a latent period, which in the case of rats is some 9–12 months. Histologically, the brain tumours are various types of glioma, whereas in the peripheral nervous system malignant neurilemmomas generally develop. Besides these types of tumour, mammary tumours and Wilm's tumours develop. The neurogenic and Wilm's tumours bear a close histological resemblance to those that form in human offspring.

These findings are of obvious significance in the aetiology of cancer in children, particularly since about one third of all malignant tumours in children originate in the nervous system or kidney. Comparative experiments in adult animals and foetuses have shown that foetal tissue is about 50 times more sensitive to carcinogenic effects than is the adult organism. Consideration must be given, therefore, to the possibility of tracing the origin of tumours, particularly of those occurring in childhood, back to the prenatal action of carcinogens.

Clinical observations in the United States (Ivankovic, *loc. cit.*) have indicated that adenocarcinomas of the vagina in young women have been caused by giving their mothers high doses of the synthetic oestrogen diethylstilboestrol, without addition of gestagen, during the pregnancy to prevent spontaneous abortion. Thus it has been established that transplacental carcinogenesis may occur in man as well as in experimental animals.

Studies in the field of transplacental carcinogenesis have yielded other findings of importance to the toxicologist. If, for example, pregnant animals are fed sodium nitrite and ethylurea, neither of which is carcinogenic when given alone, the carcinogenic ethylnitrosourea formed in the stomach is transmitted across the placenta and acts on the foetus to induce a tumour that develops significantly only in postnatal life. Future research into chemical carcinogenesis, therefore, has to take into account not only carcinogenic substances but also precursors, which by chemical reaction within the organism may form the carcinogens (N-*Nitroso Compounds Analysis and Formation*, edited by P. Bogovski, R. Preussmann and E. A. Walker; IARC Scient. Publn no. 3, Lyon, 1972). In the case of the interaction of nitrite with secondary or tertiary amines, it has been shown that the formation of carcinogenic nitrosoureas can be prevented if sufficiently high doses of ascorbic acid are added to the reaction medium (Ivankovic *et al. Naturwissenschaften* 1973, **60,** 525). Ascorbic acid, however, cannot be looked upon as a general 'anticarcinogen'; it merely prevents the endogenous synthesis of this specific carcinogen. In view of this finding, we have recommended that ascorbic acid should generally be added to products, such as tinned foods with a high nitrite content and various drugs, in which the formation of nitrosoureas seems possible. The example of ascorbic acid shows that in specific cases the formation of highly active carcinogens can now be prevented by the addition of an antidote.

The last paragraph has brought *N*-nitroso compounds into the discussion (for references see Bogovski *et al.* (eds), *loc. cit.*; Preussmann, *Handbuch allg. Path.* 1975, **6,** 421; Schmähl, 1970 *loc. cit.*). Several representatives of this group have pronounced carcinogenic effects in various animal species. Diethylnitrosamine, for example, is carcinogenic to the liver of 17 species. Several nitrosamines and nitrosamides are effective even in microgram doses if applied daily, and single applications of some nitroso compounds may lead to cancer in various organs after a latent period, which in the rat may last 1–2 years. It is particularly notable that chemically similar nitrosamides may act on different target organs, the identity of which may vary even with the dose. For example, diethylnitrosamine given to rats in a single high, but subtoxic, dose induces tumours mainly in the kidney, whereas daily doses of about 2% of the LD_{50} lead to cancer not of the kidney but of the liver, and daily doses of about 0·3% of the LD_{50} induce not only liver tumours but also oesophageal tumours in about 30% of the animals. This example illustrates the difficulty of interpreting the organotropic characteristics

of some carcinogens and relating these to carcinogenic effects in man.

In my opinion, *N*-nitroso compounds have a particular significance in relation to cancer in man, because they are found, albeit in small amounts, in the human environment and in several foods. Their action could easily explain the organ distribution of human tumours, since in experimental animals nitrosamines and nitrosamides induce tumours in organs that are frequently the site of malignant growth in man. Some will claim that nitrosamines and nitrosamides are still not proven carcinogens for man, but while this may be strictly true, these compounds represent the missing link required for an explanation of the cause of a great number of human tumours.

I have mentioned already that the target organ for the carcinogenic action of a substance may depend on whether it is administered in a single or a daily dose and have pointed out the difficulty of interpreting and predicting the specific organotropic effect of a chemical in man. The situation becomes even more difficult when small doses of different carcinogens, possibly of different chemical structure, act simultaneously on man. In this situation there are, in theory, three possibilities: additive and syncarcinogenic effects may occur, the substances may not be influenced by each other, or they may inhibit each other's carcinogenic effects.

In extensive animal experiments, dating back in some cases to 1963, we have studied the combined effects of chemical carcinogens (Schmähl, *Oncology* 1976, **33,** 73) and have proved that syncarcinogenic effects occur when chemicals possibly of different chemical structure but affecting the same target organ act simultaneously or consecutively. Syncarcinogenesis may occur even when extremely small doses of the different compounds are given. When, for example, specifically hepatotropic carcinogens are administered alone in subthreshold doses, no hepatomas develop, but if the same doses of four such substances (dimethyl- and diethylnitrosamine, nitrosomorpholine and dimethylaminobenzene) are given simultaneously, cancer of the liver develops after a precalculable latent period, indicating the existence of a syncarcinogenic effect. This shows that the so-called subthreshold doses for carcinogens are by no means harmless, since they may exert syncarcinogenic effects with other carcinogens acting on the same target organ. Similar results followed the combined applications of carcinogenic polycyclic aromatic hydrocarbons contained in tobacco smoke and polluted air. It is notable that, in the latter study, the non-carcinogenic hydrocarbons did not exert any competitive inhibitory effect on the action of the carcinogenic hydrocarbons (Schmidt *et al. Z. Krebsforsch.* 1976, **87,** 93).

The simultaneous action of carcinogens with different target organs is another problem altogether. Here mutual influences could not be detected, a logical result if one considers that the target cells attacked by these carcinogens are different. In this case, intracellular mechanisms are assumed to play a primary role in the induction of the cancer.

Of course, chemical carcinogenesis will be affected if the metabolic processes of the organism inhibit or accelerate the formation of the ultimate carcinogen. To describe this problem in greater detail would be beyond the scope of this review, but I shall deal briefly with the question of whether the organism possesses defence mechanisms against carcinogenic effects or, in other words, whether there is a relationship between the immune system in the broadest sense and carcinogenesis. This question has come to the fore recently because of two observations made in connexion with transplantation surgery. The first was the finding that after transplantation of organs from donors suffering from cancer, the recipients developed malignant tumours originating from the transplanted organ although at the time of transplantation no tumour cells were detectable macroscopically in the organ. The only possible interpretation of this fact was that owing to the immunosuppressive treatment necessary to prevent rejection, latent tumour cells present in the transplant did not die, but multiplied and led to cancer in the recipient (Penn, *Malignant Tumours in Organ Transplant Recipients*; Springer Verlag, Berlin, 1970). Similar observations made under experimental conditions had already shown that transplanted tumour cells grew faster in an organism given immunosuppressive treatment (Schmähl, 1970 *loc. cit.*). In itself, this process has nothing to do with carcinogenesis, but it shows that tumour cells may behave more or less malignantly according to the immunological status of the host. The second, even more important, observation was that a high proportion of the recipients of organ transplants developed malignant lymphomas, reticulosarcomas or solid carcinomas after strikingly short induction periods of only 1–4 years. Among these people, about 5% developed these types of tumour, an incidence some 50–100 times higher than the usual incidence in comparable age groups. Here, again, it is assumed that pre- and postoperative immunosuppression is at the root of the tumour induction (Penn, *Cancer, N.Y.* 1974, **34,** 1474). In this connexion, I should like to mention congenital immune deficiencies in children. There are increasing numbers of reports that the incidence of malignant tumours, mainly lymphomas, may be increased by as much as 10,000 times in children who suffer from overt congenital immune deficiencies (Gatti & Good, *ibid* 1971, **28,** 89).

There are two possible explanations for the formation of malignant tumours in organ-transplant recipients and children with immune insufficiency. It is possible, on the one hand, that dormant cancer cells in the body start multiplying under the influence of immunosuppressive treatment and, on the other, that immunosuppression *per se* may induce carcinogenic effects, in that unidentified surveillance mechanisms operating in the cell and controlled by the immune status of the host are destroyed. Unfortunately we have no sound evidence yet in favour of either of these theories.

Our group has studied the question of whether *chemical* carcinogenesis depends on the immune status of the host. Tests on various chemical carcinogens in a large number of animals all showed negative results, no relationship being detected between the immunological status of the host organism and chemically-induced carcinogenesis (Scherf & Schmähl, *Rec. Results Cancer Res.* 1975, **52,** 76; Schmähl, *Z. Krebsforsch.* 1974, **81,** 211;

Schmähl *et al. ibid* 1974, **82**, 91; *idem, ibid* 1976, **86**, 85 & 89). The observed influences of the immune system on human carcinogenesis seem therefore to be subject to different control and to be unconnected with chemiccal carcinogenesis. In this context, the possibility of viral carcinogenesis is worth considering. The studies briefly described here must be viewed as part of a multicomponent mechanism of carcinogenesis.

In this discussion, I have selected a few examples from the large number of chemical carcinogens in order to pinpoint the problems of chemical carcinogenesis. However, the following point is of particular importance. While it was considered until recently that carcinogenic compounds were necessarily byproducts of our technological age, various carcinogenic natural products are now known and some of these, including aflatoxins, arsenic, asbestos, betel nuts, *Cycas circinalis, Pteris aquilina*, pyrrolizidine alkaloids, streptozotocin and tobacco, are certainly or very probably carcinogenic in man. Others, notably the actinomycins, ethionine, griseofulvin, luteoskyrine, tannins (applied epicutaneously) and thiourea, are possibly carcinogens for man, but no judgement can be passed in this respect on calamus oils, *Candida parapsilosis, Claviceps purpura*, coumarin, elaiomycin, *Encephalortos Hildebrandt, Krameria ixina*, phorbol, protoanemonin, safrole, sanguinarine and sterigmatocystin, all of which have been shown to induce tumours in animals under certain conditions. All these substances, and especially some of the mycotoxins, are widespread in the environment, and in only small doses have been shown to have potent carcinogenic effects in several animal species. Some have also shown transplacental effects. The large group of naturally occurring carcinogens has been studied very little so far and offers great scope for future research.

I have tried here to point out connexions between the results of experimental cancer research and observations and experiences in human carcinogenesis. Experimental cancer research must use the results of animal experiments but although in many cases a striking conformity has been observed between experimental findings and observations in man, the results of animal studies cannot be transferred uncritically to the human situation. This does sometimes occur, often for political reasons, and beneficial substances are banished from the human environment on grounds of doubtful experimental evidence. This has been the case with some artifical sweeteners and insecticides. Although toxicology has the task of protecting the population against potential hazards, it also has a duty to weigh results critically and to consider risk-benefit ratios in judging potentially carcinogenic substances. This responsibility applies particularly to the potential carcinogenic side-effects of drugs (Schmähl *et al. Iatrogenic Carcinogenesis*; Springer Verlag, Berlin, in press).

Clearly substances must be eliminated from the human environment as far as is technically possible if they have been shown to be carcinogenic in well-conducted studies in several animal species and if frequent human contact with them is likely. Elimination of such substances from the human environment whenever possible can reduce the incidence of human tumours. The clearest examples of this, already of historical interest, are some types of occupational cancers, the incidence rates of which have been reduced by eliminating certain carcinogens from the working environment by industrial hygiene measures.

The situation becomes difficult and complex when weak carcinogens are involved or when the relevant animal studies are unsatisfactory or inappropriate. In these cases, restrictive decisions should be made only when clear results are available. Unfortunately, uncritical reporting of such cases by the mass media often forces scientists and legislative authorities to take political rather than scientific decisions. Such practices should be resisted unequivocally by toxicologists.

I hope to have shown that chemical carcinogens may be responsible for many human tumours, and to have provided some insight into the pharmacodynamic mechanisms of such carcinogens, their effects in combination and the role of the immune system in chemical carcinogenesis. We should all strive to protect the population against carcinogenic chemicals, but clearly zero values cannot be achieved and compromises have to be made. While working to protect the population against toxic hazards in the environment, the responsible toxicologist must not forget the quality of life which has become established in this century and which should be preserved and developed where possible by all of us.

CADMIUM: A SOMBRE PICTURE

Cadmium (Cd) is a toxic metal which is widespread in the environment and has a tendency to accumulate in the mammalian kidney (*Cited in F.C.T.* 1975 **13**, 470). It has been incriminated in the Japanese condition known as 'itai-itai', characterized by severe bone pain and osteomalacia, since it is a prominent contaminant of water used to irrigate rice fields (*ibid* 1972, **10**, 249). This allegation is not without its critics, however, largely because the occurrence of 'itai-itai' is far more localized than the contamination of Japanese surface waters by Cd (Nogawa *et al. Envir. Res.* 1975, **10**, 280). Attempts have continued, therefore, to settle this question, as well as to identify more clearly the various effects of known Cd exposure in man.

'Itai-itai' disease

When lymphocytes from five Swedish males exposed industrially to Cd and from four Japanese females suffering from 'itai-itai' disease were subjected to chromosomal analysis (Bui *et al. ibid* 1975, **9**, 187), there was no evidence in the Swedes of any Cd-induced chromosome damage but the 'itai-itai' patients, like a group of Japanese controls, showed

a higher frequency of chromosome breaks, but not chromatid breaks, than the Swedes. On this evidence some agent other than Cd might well be involved in the pathogenesis of 'itai-itai'. Nogawa *et al.* (*loc. cit.*) have described five 'itai-itai' patients found in the Ichi River basin, where Cd pollution has been reported. Since these patients were originally diagnosed as suffering from Fanconi syndrome they were treated with vitamin D, to which they made a satisfactory response. However, post-mortem examination of one of them 14 months after treatment with the vitamin revealed tissue-Cd concentrations raised to levels similar to those found in 'itai-itai' patients, and the conclusion was reached that a diagnosis of 'itai-itai' induced by exposure to environmental Cd was probably more accurate in these cases than one of Fanconi syndrome, which includes similar disturbances of calcium metabolism.

The relatively high Cd content of the average Japanese diet has been matched by determinations of tissue concentrations of zinc and Cd in whole embryos of the first trimester and in the liver, kidney and brain of foetuses of the second trimester derived from Japanese mothers (Chaube *et al. Archs envir. Hlth* 1973, **26,** 237). All of the foetuses were grossly normal and none of the mothers had 'itai-itai' or lived in an area where it had been found. In first-trimester tissues, Cd was present in concentrations of 0·032–0·07 μg/g wet tissue in 57% of embryos. In second-trimester tissues, it was present in 80% of livers (at a mean level of 0·113 μg/g), in 28% of kidneys (mean 0·05 μg/g) and in 17% of brains (mean 0·14 μg/g). Since Cd is known to act as an antimetabolite of zinc, which plays an essential role in growth and development, such concentrations of Cd may be of considerable significance in the development of the infant.

Lungs and kidneys in occupational exposure

Many recent studies have been concerned with occupational exposure and with the re-examination of the TLV for Cd dusts in working atmospheres. Lauwerys *et al.* (*Archs envir. Hlth* 1974, **28,** 145) studied groups from three different factories making or using Cd and classified them into women with less than 20 years' exposure to Cd dust, men with less than 20 years' exposure, and men with more than 20 years' exposure. The concentrations of airborne Cd dust sampled in the factory areas during the study were below the then accepted maximum of 200 μg/m^3 and no major plant modifications likely to have affected the dust levels substantially had occurred since installation. Nevertheless, men exposed for longer than 20 years showed a slight but significant reduction in forced vital capacity (FVC), forced expiratory volume at 1 second (FEV_1) and peak expiratory flow rate. Excessive proteinuria was observed in 68% of this group, as well as in 15% of the men exposed to Cd for less than 20 years, signifying that the kidney is more susceptible to Cd intoxication than the lung. The electrophoretic pattern of the urinary proteins in the affected males suggested that, in Cd-induced kidney damage, an increase in glomerular permeability precedes the development of a predominantly tubular lesion and may therefore serve as a warning sign. In the women, who were exposed for an average of 4 years, the only index that differed significantly from that in appropriate controls was the urinary level of Cd. The observations in this study provided support for a proposal that the TLV for Cd dust be lowered from 200 to 50 μg/m^3, a move that was put into effect in the USA in the following year and is now being reconsidered, with the likelihood of a further reduction to 40 μg/m^3 (*Federal Register* 1977, **42,** 5434). On the basis of a demonstrated correlation between proteinuria and urinary Cd levels, Lauwerys *et al.* (*loc. cit.*) suggested that the concentration of Cd in the urine of exposed workers should be kept below a threshold of 15 μg/g creatinine, at which level detectable renal damage is unlikely.

A reduction in FVC, but not in FEV_1 or expiratory flow was demonstrated by Smith *et al.* (*Am. Rev. resp. Dis.* 1976, **114,** 161) in workers exposed for long periods (6–40 years) in areas where the concentrations of Cd (mainly as freshly generated Cd fume) commonly exceeded 200 μg/m^3. Urinary Cd concentrations in this group (45·7 μg Cd/litre on average) were slightly higher than those reported by Lauwerys *et al.* (*loc. cit.*), who found an average of 39 μg/litre (assuming 1·25 g creatinine/litre at a specific gravity of 1·018). The respiratory-function impairment and chest X-rays reported by Smith *et al.* (*loc. cit.*) suggested a mild fibrotic reaction following long-term Cd exposure. These authors reported a correlation between the reduction in FVC and the months of work in Cd-fume areas, but there was no such correlation for exposure to Cd sulphate aerosol.

Biochemical investigation of 27 coppersmiths exposed to fumes from brazing for an average of 17·8 years indicated that such workers had a greater tendency to suffer liver damage, renal impairment and restrictive airway disease than controls. Among these 27 workers, Scott *et al.* (*Lancet* 1976, **II,** 396) found raised plasma levels of urea in 20, of creatinine in 13 and of chloride in 13. A reduced plasma phosphate level in 13 was a further indication of possible damage to the kidney tubules. In 11 of the men, proteinuria was significant, and radiological examination confirmed renal calculi in five. The FEV/FVC ratio was below 75% in 18 of the workers and of these, nine showed evidence of restrictive airway disease, four of obstructive airway disease, and one of both. No correlation of these defects with smoking habits could be established. Morgan (*ibid* 1976, **II,** 585) criticized these findings as introducing confusion between restrictive and obstructive airway disease, and claimed that pulmonary impairment after exposure to Cd had not been established.

A recently published report (Kolonel, *Cancer, N.Y.* 1976, **37,** 1782) has suggested that occupational exposure to Cd could perhaps be a factor not only in the impairment of renal tubular function but also in the development of renal cancer. So far there is little evidence for any true carcinogenicity on the part of Cd in experimental animals, let alone in man (*Cited in F.C.T.* 1972, **10,** 251), but it is known that Cd accumulates in the human renal cortex throughout the greater part of life. Surveys carried out in Sweden (Elinder *et al. Archs envir. Hlth* 1976, **31,** 292; Piscator & Lind, *ibid* 1972, **24,** 426) have confirmed earlier findings in the United States that Cd levels in the

renal cortex increase steadily from birth until the age of about 40–50 years, and then begin to decline again. This pattern of accumulation in the kidney is paralleled by that of zinc, although in most parts of the body the zinc content is almost constant throughout life. Kolonel (*loc. cit.*) based his hypothesis on a retrospective investigation of the types of Cd exposure to which 64 New York males suffering from renal cancer had been subjected. The information was compared with that for two other groups of males from the same age group (50–79 years), who were admitted to the same hospital during the 1957–1964 study period suffering from malignant tumours of the colon in the one case and non-malignant diseases of the digestive system in the other. This study was necessarily fairly small, since malignant renal tumours are relatively uncommon in human adults, but the results from this limited sample showed a significant association between renal cancer and occupational exposure, the relative risk of renal cancer in men so exposed being some 2·5 times greater compared with either the non-cancer or cancer control group. No significant risk was associated with Cd exposure derived from cigarette smoking, but for those who smoked and were occupationally exposed to Cd the relative risk was more than four times as great as the risk for those who fell into neither category. This apparently synergistic rather than additive effect suggests, however, that any contributing factor in cigarette smoke would probably be some component other than Cd. The study thus suggests the possibility that the previously reported association between renal cancer and smoking could perhaps be a matter of synergism between smoking and exposure to other sources of Cd, including the diet, but clearly a great deal more work on this question will be essential before any firm conclusions can be drawn.

Hypertension

The increase of man's normal body burden of Cd by cigarette smoking also complicates the already controversial subject of the relationship between Cd and hypertension. This is an interesting topic, since although hypertension is not one of the established signs of cases of Cd poisoning, it does follow the ingestion of very small amounts of Cd by rats (*Cited in F.C.T.* 1966, **4,** 203), and in many hypertensive patients the Cd content of the kidneys (*ibid* 1966, **4,** 203) and blood (Glauser *et al. Lancet* 1976, **I,** 717) has been found to be unusually high. The latter authors reported a mean blood-Cd concentration of 3·4 ng/ml in healthy normotensive subjects compared with one of 11·1 ng/ml in matched untreated hypertensive patients. All the normotensive subjects had blood-Cd levels below 8 ng/ml, while in 13 of 17 hypertensives this concentration was exceeded. Piscator (*ibid* 1976, **II,** 370) criticized these observations on the ground that insufficient consideration had been paid to other possible factors in hypertension, particularly the smoking habit. Glauser (*ibid* 1976, **II,** 371) countered this charge by pointing out that a significant fall in blood-Cd concentration, bringing the level to within normal limits, had been observed in patients whose blood pressure was reduced by a thiazide diuretic, without any concurrent alteration of smoking habits. However, Ulander & Axelson (*ibid* 1974, **i,** 682) reported a great difference in blood-Cd concentrations between smokers and non-smokers, although it was not clear whether the raised blood levels reflected the accumulation of Cd in the tissues or the current uptake from tobacco.

More recently, Beevers *et al.* (*ibid* 1976, **II,** 1222) have provided confirmation of a significantly higher blood-Cd level in smokers than in non-smokers, but their study, in which 70 hypertensive patients in Renfrew, Scotland, were compared with 70 matched normotensive controls, failed to demonstrate any relationship between blood-Cd levels and hypertension. It is suggested that one reason for the difference between these results and those of the smaller study of Glauser *et al.* (*loc. cit.*) may be the use of antihypertensive treatment by many of the patients in the Scottish study, although there were no differences between the Cd levels in treated and untreated patients or in patients on widely differing treatments. The role of Cd in the development of hypertension thus remains an open question (which we hope to discuss in more detail in a future issue). More clear is the contribution of smoking to the overall body burden of Cd, and Piscator *et al.* (*Lancet* 1976, **II,** 587) have warned that this situation may be exacerbated when smoking is permitted in some factory areas. They reported that handling by workers in a plant making nickel–Cd batteries could increase the Cd content of cigarettes from 1·3–1.8 μg/g to as much at 26 μg/g and of pipe tobacco from 1·7–9·1 μg/g to 20–315 μg/g.

Measurement of Cd in tissues

The choice of blood levels as an index of the body burden of Cd in the studies on hypertension mentioned above was criticized by Morgan (*ibid* 1976, **II,** 1361), who pointed out that blood contains only about 0·1% of the total body burden and that neither blood levels nor urinary excretion correlate well with the amounts in kidney and liver, which together account for about half of the body burden. Analyses of tissue levels, while more relevant than those of blood and urine, are also more difficult, but the accurate measurement of traces of Cd in tissues is now possible by means of neutron-activation analysis. This technique was developed by McLellan *et al.* (*Physics Med. Biol.* 1975, **20,** 88) for the screening of industrial workers at risk from Cd exposure and has a limit of sensitivity, established in a liver-size phantom, of 0·5 ppm for a dose of 0·4 rad. Application of this technique to the estimation of Cd in the livers of four men with known or suspected Cd poisoning revealed concentrations of 35–200 ppm Cd, in contrast to levels of less than 1 ppm in non-exposed subjects. Perhaps the application of this technique to the problem of the role of Cd in hypertension would allow more progress to be made.

[P. Cooper—BIBRA]

DIOXIN: A NEW BIOLOGICAL PROBE?

An explosion in a trichlorophenol plant in a North Italian town in July 1976 turned 'Seveso' and, soon after, 'dioxin' into household words. 'Dioxin' or 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) is reputed to be one of the most toxic compounds known. It arises as an impurity during the manufacture of 2,4,5-trichlorophenol by alkaline hydrolysis and is therefore a potential contaminant of commercial 2,4,5-trichlorophenoxyacetic acid (2,4,5-T). The explosion at Seveso resulted in the deposition of both trichlorophenol and TCDD dust over a populated area south of the factory and led subsequently to the wholesale evacuation of the local inhabitants, to the widespread destruction of crops and deaths among the local animal populations and to the instititution of long-term decontamination efforts. The first effects on the human population were skin rashes in children, and within a few weeks many of the 500 people reported to be undergoing treatment for poisoning were showing signs of kidney and liver malfunction, while others were suffering from chloracne (Hay, *Nature, Lond.* 1976, **262,** 636). Chloracne is a well-known effect of TCDD (*Cited in F.C.T.* 1974, **12,** 274). In addition, porphyria, probably associated with liver damage, has been observed in affected mice, and liver hypertrophy and thymic regression in rats (Allen *et al. Fd Cosmet. Toxicol.* 1975, **13,** 501; *Cited in F.C.T.* 1975, **13,** 586), and the compound also has teratogenic potential (*ibid* 1972. **10,** 722; *ibid* 1974, **12,** 418). Some 7 months after the explosion, the latter aspect was still a matter for speculation and concern, and considerable numbers of schoolchildren were developing chloracne (Hay. *Nature, Lond.* 1977, **266,** 7).

A major problem with TCDD is its capacity to induce delayed effects. These do not always occur (Beale *et al. Lancet* 1977, **I,** 748), but they featured in a report concerning three young men who suffered transient and mild exposure (Oliver, *Br. J. ind. Med.* 1975, **32,** 49). Two of these men were working in separate laboratories on the preparation of TCDD for laboratory standards, and despite carrying out their work in a fume cupboard and wearing overalls and rubber gloves, they developed chloracne, typically affecting the face, ears and neck. One of these two and a colleague working with the diluted standard developed delayed reactions some 2 years after the exposure. These included colicky abdominal pain with flatulence intensified by farinaceous foods, headache, irritability, loss of vigour, excessive fatigue, difficulty in muscular and mental co-ordination, and excessive growth of dark hair on the shoulders. These delayed effects did not occur in the other chloracne sufferer, while the second man with delayed symptoms had no chloracne. In all three men, however, serum cholesterol concentrations were high, exceeding 300 mg/100 ml when a check was made some 3 years after exposure, by which time the other effects had largely receded. No liver damage or prophyrinuria was detected. The suggestion is put forward that the cluster of effects may possibly represent some hormonal disturbance induced by TCDD, affecting sebaceous secretion, blood-cholesterol metabolism and hair growth.

Experimental studies

Male mice given 250 μg TCDD/kg by mouth were examined after 1–35 days (Jones & Greig, *Experientia* 1975, **31,** 1315). At day 8 or later the livers of treated animals were larger and paler than those of controls, and some moribund mice showed subcutaneous oedema and bleeding into the gastro-intestinal tract. During the first 16 days after dosing, progressive centrilobular hepatic necrosis developed. Eosinophilia of centrilobular cells was evident at 24 hours and after 5 days infiltration by mononuclear cells and a few polymorphonuclear leucocytes was apparent, with inflammatory cells and connective tissue forming bands linking adjacent centrilobular areas. At 12–16 days, mononuclear cells had infiltrated the portal tracts and sinusoids, and proliferation of bile ducts and connective tissue had occurred. Lipid vacuolation was apparent in the parenchymal cells of all zones by day 16. By day 35 inflammation had largely disappeared but fibrosis persisted in the larger portal tracts. Part at least of the increase in liver weight was attributed to the increase in lipid content. Liver DNA content was unaltered, but the liver cholesterol level rose.

The same group showed that rats given an oral dose of 200 μg TCDD/kg developed liver damage, which was again largely confined to the centrilobular zone (Jones & Butler, *J. Path.* 1974, **112,** 93). Necrosis of parenchymal cells and mononuclear cell infiltration were evident after 1 week and an occasional large parenchymal cell with four to six nuclei was present. After 2 weeks the sinusoids appeared dilated and the inflammatory reaction was more marked. During the next 4 weeks, the latter lesion regressed and the multinucleate parenchymal cells increased in size and formed ring structures, but they showed no mitotic figures. Repair of the liver lesion, with increasing numbers of normal mitotic figures, was apparent during weeks 6–9. Histochemical studies on liver slices from rats given 200 μg TCDD/kg showed a significant alteration of adenosine-triphosphatase (ATPase) reaction on day 3, with loss of reactivity within an area five or six cells deep surrounding the central vein (Jones, *ibid* 1975, **116,** 101). This abnornal distribution of ATPase was clearly defined at 5–8 days, and the extensive loss of enzyme persisted after 42 days. A normal ATPase reaction and partial restoration of the liver structure was seen in rats killed 9 months after treatment. Qualitative changes in 5-nucleotidase and acid phosphatase appeared to be secondary to parenchymal-cell damage. Both the morphological and histochemical findings in this study thus point to the parenchymal-cell plasma membrane as the specific subcellular site of TCDD toxicity.

A report by Jackson (*J. Cell Sci.* 1972, **10,** 15) that TCDD in concentrations of about 0·1 μg/litre of culture medium inhibited mitosis in rapidly dividing endosperm cells of the African blood lily prompted a number of studies on the compound's possible effect on mitosis in various types of mammalian cell. Beatty *et al.* (*Toxic. appl. Pharmac.* 1975, **31,** 309) failed to detect any effects on cell growth or morphology in Hela cells, normal mouse fibroblasts (BALB-3T3), a virus-transformed derivative of mouse fibroblasts (SV101), normal human foreskin fibroblasts or nor-

mal human lymphocytes grown in culture, although incorporation of TCDD into the cells was confirmed in human fibroblasts and SV101 cells by incubation with ^{14}C-labelled TCDD. It was suggested that this lack of effect might have been a reflection of the specificity of TCDD activity for cell types other than those studied.

Free from this possible drawback, in view of the *in vivo* studies mentioned above, were attempts by Greig *et al.* (*Chemico-Biol. Interactions* 1974, **8**, 31) and by Conaway & Matsumura (*Bull. env. contam. & Toxicol.* (*U.S.*) 1975, **13**, 52) to determine whether TCDD affected nucleic acid synthesis in the rat liver. The former group used partial (70%) hepatectomy to stimulate mitosis in the liver cells, since it was felt that a rapidly dividing system might be more sensitive to the action of TCDD. Nevertheless, they failed to demonstrate *in vivo* any significant effect on DNA synthesis, as measured by [^{3}H]thymidine incorporation 24 hours after hepatectomy, in the livers of rats treated with TCDD in a dose of 10 μg/kg ip or 10 or 200 μg/kg by gavage 0–72 hours before the operation, although the rate of DNA synthesis was always lower in the TCDD-treated groups than in the controls. However, when mitosis was stimulated in the rat kidney by treatment with folic acid or lead acetate, a prior dose of 10 μg TCDD/kg given ip or 200 μg/kg given orally depressed DNA synthesis by nearly 50%. Further experiments indicated that this ability of TCDD to reduce the capacity of cells of the renal tubular epithelium, and perhaps also of the liver, to initiate DNA synthesis in response to a proliferative stimulus was not due to the direct interaction of TCDD with DNA, to inhibition of protein synthesis, to interference with the time course of the mitotic cycle or to a reduction in food intake, but the authors did not rule out the possibility of some microsomal-enzyme involvement.

On the other hand, Conaway & Matsumura (*loc. cit.*) reported that when liver slices taken from rats treated 10 days previously with a single oral dose of only 5 μg TCDD/kg were incubated with [*Me*-^{3}H]-thymidine or [5-^{3}H]uridine, the incorporation of [^{3}H]thymidine but not that of [^{3}H]uridine into the hepatic nuclei was significantly higher than that in control livers. The major part of the radioactivity and the greatest difference in [^{3}H]thymidine uptakes between TCDD-exposed and control livers were located in the chromatin rather than in the nuclear membrane, indicating an intranuclear site for TCDD activity. These authors seem to favour a direct interference by TCDD in DNA synthesis and in discussing this possibility refer to a report by Vos *et al.* (*Envir. Hlth Perspec.* 1973, no. 5, 149) that sublethal doses of TCDD suppressed cell-mediated immunity and the total number of leucocytes and lymphocytes formed in the guinea-pig. Subsequently Vos & Moore (*Int. Archs Allergy appl. Immun.* 1974, **47**, 777) reported impaired cellular immunity and a severe depletion of lymphocytes in the thymic cortex of the offspring of rats and mice that had been treated with 5 μg TCDD/kg during the latter half of gestation.

Distribution and excretion

In rats fed [^{14}C]TCDD at a dietary level of 7 or 20 ppb (b = 10^9) for 42 days (Fries & Marrow, *J. agric Fd Chem.* 1975, **23**, 265), food consumption and growth were more depressed in males than in females, but recovery followed discontinuation of the TCDD treatment. There was an increase in the liver-to-body weight ratio, but this was both greater and more readily reversible in the lower exposure group. Tissue TCDD concentrations were highest in the liver (accounting for 85% of the total body burden in males and 70% in females). In other tissues the residue was 2–3 times higher in females than in males, but this difference was largely eliminated when the TCDD concentration was expressed in terms of body fat. Total TCDD retention in the body rose from 5·5 times the daily intake at 14 days to 10 times the daily intake at 42 days. Rates of residue elimination from males and females gave half-lives of 12 and 15 days respectively.

Rats given [^{14}C]TCDD in a single oral dose of 1 μg/kg showed radioactivity in the faeces but not in the urine or expired air (Rose *et al. Toxic. appl. Pharmac.* 1976, **36**, 209). When doses of 0·01–1 μg/kg/day were given on 5 days/week for 7 weeks, however, a significant amount of activity appeared also in the urine (3–18% of the cumulative dose by week 7), with females excreting a mean of 12·5% of the cumulative dose and males 3·1% at the 1-μg/kg/day dose level. Calculated steady-state values for ^{14}C in whole body, liver and fat were approached at similar rates within 13 weeks, irrespective of the daily dose.

Enzyme-inducing activity

When introduced into the air sac of fertilized hen eggs between days 15 and 20 of incubation, TCDD proved a potent inducer of aryl hydrocarbon hydroxylase activity in the chick-embryo liver (Poland & Glover, *Molec. Pharmacol.* 1973, **9**, 736). At the lowest test dose of 0·5 ng TCDD/egg (1·55 pmol/egg), hydroxylase activity nearly doubled, while at 50 ng/egg it showed a tenfold increase. Enzyme induction by TCDD persisted for at least 5 days, in contrast to the relatively brief induction by 3-methylcholanthrene (3-MC), and the potency of TCDD as a hydroxylase inducer was at least 1000 times the potency of other halogenated dibenzo-*p*-dioxins tested. At 9·6 ng/egg, TCDD also substantially increased δ-aminolaevulinic acid synthetase activity, being several hundred times more active in this respect than other halogenated dibenzo-*p*-dioxins. In another study (*idem, ibid* 1974, **10**, 349), rats and mice received a single ip injection of TCDD, and microsomal liver preparations were then examined for oxygenase and cytochrome *P*-450 activity. At 0·31 nmol TCDD/kg, aryl hydrocarbon hydroxylase activity increased fivefold, while at 31 nmol/kg induction was maximal, TCDD showing in this case nearly 30,000 times the potency of 3-MC on a molar basis. Both inducers produced a spectrally distinct type of cytochrome *P*-450. At the 31-nmol/kg level, TCDD elevated aryl hydrocarbon hydroxylase activity and microsomal haemoprotein for longer than 35 days, while aminopyrine *N*-demethylase and NADPH-cytochrome c reductase were little affected.

Subsequently, Poland & Glover (*ibid* 1975, **11**, 389) showed that while mice could be divided into responsive strains (C57BL/6J, BALB/cJ and A/J) and unresponsive strains (DBA/2J, AKR/J and SJL/J), on the

basis of the presence or absence of a response to the enzyme-inducing activity of 3-MC, both groups responded to TCDD. However, the TCDD dose required for half-maximal induction of aryl hydrocarbon hydroxylase was about 1 nmol/kg in the responsive strains and at least 10 nmol/kg in the unresponsive. Challenge with TCDD at 3 nmol/kg served to differentiate the two types. Heterozygous offspring of C57BL/6J and DBA/2J parents showed an intermediate sensitivity towards TCDD.

Data published recently by this group (Poland *et al. J. biol. Chem.* 1976, **251,** 4936; Poland & Kende, *Fedn Proc. Fedn Am. Socs exp. Biol.* 1976, **35,** 2404) support the hypothesis that this absence of response to the aryl hydrocarbon hydroxylase-inducing activity of 3-MC and other polycyclic aromatic hydrocarbons in certain strains of mice and the reduced sensitivity to induction by TCDD in the same strains is due to a mutation which results in an induction receptor with a diminished affinity for the inducing compound. These data suggest that high affinity sites, which stereospecifically and reversibly bind TCDD, are present in the cytosol fraction of the liver in responsive strains of mice (and rats) and form the receptor for the induction of hydroxylase activity. Polycyclic hydrocarbon inducers of hydroxylase activity compete with TCDD for these binding sites, but in nonresponsive species the receptor protein is altered and has a diminished affinity for the inducers.

In other studies on the induction of aryl hydrocarbon hydroxylase activity by TCDD, Niwa *et al.* (*Molec. Pharmacol.* 1975, **11,** 399) demonstrated in a wide variety of cell cultures that the inducing activity of TCDD was some 250–900 times as great as that of 3-MC, and was inhibited by actinomycin D and cycloheximide, but they found no relationship between the degree of induction and the cytotoxicity of TCDD. Hook *et al.* (*Chemico-Biol. Interactions* 1975, **10,** 199; *Biochem. Pharmac.* 1975, **24,** 335) have pointed out the marked differences in the responses of rats, guinea-pigs and rabbits and of rats of different age and sex to the enzyme-inducing activities of TCDD in various tissues, while Lucier *et al.* (*ibid* 1975, **24,** 325) have reported in detail on the induction of hepatic glucuronyltransferases. These enzymes are important in the metabolic regulation of many steroid compounds as well as in the metabolism and excretion of xenobiotics, but while a marked increase in the rate of *p*-nitrophenol glucuronidation was demonstrated in microsomal preparations from rats treated with TCDD, no effect on steroid glucuronidation was detected in the same animals.

The potent and very persistent stimulation of microsomal enzymes by TCDD in adult rats and mice and the known teratogenicity of the compound prompted a perinatal study of these enzymes in rats (*idem, Chemico-Biol. Interactions* 1975 **11,** 15) following administration of a subteratogenic dose of TCDD (3 μg/kg) during early, middle or late pregnancy (on day 5, 10 or 16). Increases in the activity of some maternal hepatic microsomal enzymes persisted for at least 10 weeks. Foetal glucuronidation of testosterone and *p*-nitrophenol was not altered, but in late gestation the activity of benzopyrene hydroxylase in the foetus was increased. Day-old offspring showed marked elevations of *p*-nitrophenol glucuronyltransferase, benzopyrene hydroxylase and cytochromes *P*-450 and b_5. Glucuronidation of *p*-nitrophenol exceeded the control value by a factor of 8 in 3-week-old pups and 8 weeks after birth the rate was still double that in the controls. Benzopyrene hydroxylation reached its peak (20 times the control value) 1 day after birth. The postnatal effect of TCDD appears to result both from ingestion of the toxin in maternal milk and, to a lesser extent, from postnatal activation of an independent inducing mechanism.

The various studies considered here support the view that TCDD possesses unique toxicological features. It is a potent toxin, yet the first effects appear only after several days, and although it is a lipid-soluble compound it apparently accumulates mainly in the liver rather than in the fat depots. Moreover, the pathological picture it induces in the liver is not produced by any other known chemical. The large multinucleate hepatocytes seen in rats poisoned with TCDD are, instead, strongly reminiscent of the lesion seen in neonatal hepatitis, a disease induced by the infective hepatitis virus; this lesion is unique in human pathology.

[P. Cooper—BIBRA]

TRANSFORMATION *IN VITRO*—AN INDEX OF CARCINOGENICITY?

In the living animal, one important characteristic distinguishes cancer cells from normal cells, namely their capacity for uncontrolled proliferation. This unusual ability of continued multiplication is also seen *in vitro*. Under these conditions, cancer cells continue to multiply indefinitely, producing multilayered sheets of cells, in contrast to non-cancer cells which produce only a single sheet.

This property is reflected in the ability of malignant cells to form colonies when inoculated into soft agar; non-malignant cells survive under these conditions but do not multiply to form colonies. However, when cultures of normal mammalian cells are infected with oncogenic viruses (which induce cancer when inoculated in experimental animals, such as chickens or mice), the cells acquire the ability to form colonies in soft agar and to produce multilayered sheets. In the terminology of the oncologist, viruses have transformed the normal cells into cancer cells, and indeed, inoculation of cultures of such transformed cells into suitable animals results in the development of a tumour at the site of inoculation.

Transformation *in vitro* can also be brought about by chemical carcinogens, but the transformed cells produce tumours much less consistently when inoculated into experimental animals than do virus-transformed cells. Despite this obvious shortcoming, cell transformation *in vitro* is now being advanced as a means of detecting the potential carcinogenicity of chemicals (Purchase *et al. Nature, Lond.* 1976, **264,**

624). Since, according to current thinking, chemical carcinogens are also mutagens, there are some grounds for considering that the new growth potential of cells following their treatment with a chemical carcinogen may be indicative of a change in the genetic material of the cell. Moreover, there is some experimental evidence to support this hypothesis (Bouck & di Mayorca, *ibid* 1976, **264**, 722).

More clearly indicated, however, is an association between *in vitro* cell transformation and a reduction in the major protein component of the cell membrane, a protein with a molecular weight of 250,000 and generally known as 'LETS' (Vaheri & Ruoslahti, *Int. J. Cancer* 1974, **13**, 579). LETS is present on the surface of fibroblasts from a variety of species. It decreases or disappears when the cells are transformed by viruses, but it remains to be established whether this is because the cells fail to synthesize the protein, because the protein fails to be inserted after synthesis or because it is broken down by proteolytic enzymes after binding. Whatever the underlying cause, the loss of LETS from the cell surface is regarded as one important consequence of cell transformation (Blumberg *et al. Nature, Lond.* 1976, **264**, 446).

Apart from the question of virus infection, it has been shown recently that the amount of LETS in the cell membrane can be reduced considerably by treating cultures of chick-embryo fibroblasts with phorbol myristate acetate (PMA). This observation is important because PMA is one of several phorbol esters that are found in croton oil and are potent tumour promoters, although they show little direct carcinogenic activity. Such esters have been shown to transform mouse fibroblasts previously irradiated with ultraviolet light (Mondal & Heidelberger, *ibid* 1976, **260**, 170), although at the levels of exposure used, neither the irradiation nor the phorbol ester was active alone in transforming the cells. The addition of 10 or 100 ng PMA/ml to secondary chick-embryo fibroblasts was found to result within 3 days in a 75% reduction in the amount of LETS on the cell membrane (Blumberg *et al. loc. cit.*). When the cells were transferred after this period to a PMA-free medium they regained the lost LETS within a further 3 days. This sequence of events was the same as that demonstrated in similar cultures treated with Rous sarcoma virus, but the virus-transformed cells lost LETS at a much faster rate and also recovered it more quickly when the virus was removed by appropriate treatment.

Other evidence of the ability of phorbol esters to alter the cell membrane has been presented by Wenner *et al.* (*Cancer Res.* 1974, **34**, 1731), who showed that 12-*O*-tetradecanoyl phorbol 13-acetate (TPA) reduced the electrophoretic mobility of hyperdiploid Ehrlich-Lettré ascites tumour cells (ELD). Like PMA, this ester is known to be an active tumour-promoting agent. Comparable concentrations of an inactive phorbol stereoisomer (4α-phorbol didecanoate) did not reduce the mobility of ELD.

Thus, those phorbol esters that are known to be active tumour-promoting agents clearly have an effect on the cell membrane. Attempts to identify their effect on DNA have indicated that, in keeping with their capacity to induce cell multiplication, they stimulate DNA synthesis and ornithine-decarboxylase activity in mouse epidermal cells in culture to a degree that seems to be proportional to their tumour-promoting ability (Yuspa *et al. Nature, Lond.* 1976, **262**, 402). On the other hand, they do not induce unscheduled DNA synthesis or inhibit DNA repair (Trosko *et al. Chemico-Biol. Interactions* 1975, **11**, 191) and in these respects they differ fundamentally from carcinogens (San & Stich, *Int. J. Cancer* 1975, **16**, 284).

Clearly a demonstration of cell transformation *in vitro* need not necessarily indicate a genetic change but may be a reflection of damage to the cell membrane and if cell transformation is to be used as a basis for a short-term test for carcinogens, it is essential to differentiate between these two mechanisms. Drawing this distinction may open the way for the development of techniques capable of identifying promoters as well as carcinogens.

[P. Grasso—BIBRA]

HEPATOTOXIC ANIT

α-Naphthylisothiocyanate (ANIT), a compound with insecticidal properties, produces marked changes in liver function in mammals, notably bilirubin retention and reduced bile flow (*Cited in F.C.T.* 1974, **12**, 161). In this respect ANIT somewhat resembles carbon disulphide (CS_2), but unlike CS_2 it increases the serum activity of glutamic–pyruvic transaminase and its effects are potentiated by phenobarbitone (*ibid* 1973, **11**, 339). More details of its mode of action are now available.

The histological effects of ANIT on the liver have been studied by Ungar & Popp (*Archs Path.* 1976, **100**, 127). Male mice given a single oral dose of 50 or 100 mg ANIT/kg, or similar doses of *p*-phenylenediisothiocyanate (PDT), were killed for liver examination after 1, 2, 4 and 7 days. Those given ANIT were jaundiced at 24 and 48 hours and those taking 100 mg/kg remained jaundiced on day 4. At 100 mg/kg, PDT also induced jaundice, which persisted until day 7, but a dose of 50 mg/kg was without icteric effect. Histological studies showed that 24 hours after a dose of 100 mg ANIT/kg, intrahepatic bile ducts were denuded of epithelium and their lumens were filled with debris, while the adjacent parenchyma was necrotic. The gall bladder in these animals was distended and its mucosa was divested of folds, while the submucosa was hyperaemic and oedematous. After 48 hours, the liver changes included periportal infiltration of lymphoid cells. In mice given 50 mg ANIT/kg, the hepatocyte necrosis was minimal and very restricted in area, and gall-bladder lesions did not appear. By day 4 the biliary-tract epithelium had completely regenerated and the gall-bladder mucosa had recovered regular folds. By day 7, few foci of necrosis remained in the liver and the intrahepatic bile ducts had regenerated, while the gall bladder had regained a normal appearance. A similar but more intense picture of liver toxicity, with extensive

haemorrhagic parenchymal necrosis, appeared in animals given 100 mg PDT/kg; those given 50 mg PDT/kg also developed severe ductular and cellular necrosis after 48 hours.

Several different approaches have been used in attempts to elucidate the biochemical effects of ANIT. A single oral dose of 100 mg/kg was followed in rats by an increase in conjugated bilirubinaemia, which reached a peak at 48 hours and had subsided by day 7 (Hertzog *et al. Pathology* 1975, **7**, 13). At 48 hours the activities of Mg^{2+}- and Na^{+}/K^{+}-stimulated adenosine triphosphatases (ATPases) and of 5′-nucleotidase in plasma were reduced, but these reverted to the normal range by day 7. In histochemical studies, most canaliculi in the liver cortex showed variable degrees of reduction in Mg^{2+}-ATPase activity, but some retained normal activity. The indications are that ANIT toxicity involves the development of a membrane lesion which causes the observed hyperbilirubinaemia by impeding secretion into the canaliculi.

Labelling of ANIT with ^{3}H in position 4 of the naphthalene ring and with ^{14}C in the isothiocyanate group was used to determine whether the effects are caused directly by ANIT or are mediated by a toxic metabolite (Skelton *et al. Expl mol. Path.* 1975, **23**, 171). In rats treated with a mixture of ^{14}C- and ^{3}H-labelled ANIT, in which the ^{3}H:^{14}C ratio was 6·3, a relatively higher proportion of ^{3}H than ^{14}C was excreted into the bile during the first 8 hours, the ratio being increased to 7·5. This relative increase in ^{3}H excretion was blocked, however, by treatment with cycloheximide, actinomycin D or DL-ethionine, each of which have been shown to prevent or depress ANIT-induced hyperbilirubinaemia. To be effective, it was necessary for this treatment to be given several hours before or, in the case of cycloheximide, not more than 1 hour after ANIT administration. It seems, therefore, that these three agents probably suppress the development of hyperbilirubinaemia and cholestasis by inhibiting the formation of a toxic ANIT metabolite.

Other work has demonstrated, in turn, the effect of ANIT on ethionine metabolism. Female rats that had been fed 0·1% ANIT in the diet for 6–14 days showed an altered capacity for metabolizing a single oral dose of ^{14}C-labelled L-ethionine (Brada & Bulba, *Res. Commun. chem. Path. Pharmac.* 1976, **13**, 19), the excretion of free ethionine and particularly of *S*-adenosylethionine being increased at the expense of total ethioninesulphoxide. When the ethionine dose was given after ANIT feeding had been continued for up to 99 days, the excretion of free ethionine and *S*-adenosylethionine was close to normal levels, as was that of total ethioninesulphoxide, although the proportion of free as opposed to acetylated ethioninesulphoxide was much higher than in the controls. No direct correlation was observed between the alteration in ethionine metabolism and changes in the structure of the liver parenchyma. These findings suggest that the functional integrity of the hepatocytes is the major factor in the maintenance of normal ethionine metabolism.

The influence of cholestasis on microsomal-enzyme systems was studied by Drew & Priestly (*Toxic. appl. Pharmac.* 1976, **35**, 491), who gave rats 200 mg ANIT/kg by gavage and measured bile flow through a cannula to determine the onset and time course of stasis. Bile flow was not significantly reduced during the first 12 hours, but stasis was complete by 24 hours and was completely reversed 7 days later. Fractionation of liver homogenates prepared after 12 and 24 hours showed that the 10,000-g supernatant had impaired amidopyrine- and aniline-hydroxylating capacity at 12 hours, while the 105,000-g microsomal fraction showed no such impairment until cholestasis had become established at 24 hours. Bile stasis appeared to result in reversible hypoactivity of the smooth endoplasmic reticulum. Once cholestasis had become established, the level of bilirubin and the activity of 5′-nucleotidase in plasma increased, but as soon as bile flow had been restored these plasma components fell again to their normal limits.

[P. Cooper—BIBRA]

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TOXICOLOGY: BIBRA ABSTRACTS AND COMMENTS

COLOURING MATTERS

3194. Postscript on amaranth?

Khera, K. S., Roberts, G., Trivett, G., Terry, G. & Whalen, C. (1976). A teratogenicity study with amaranth in cats. *Toxic. appl. Pharmac.* **38,** 389.

A suggestion (Drake, *Fd Cosmet. Toxicol.* 1977, **15,** 153) that the last word might have been expressed on amaranth teratology has proved premature. No sooner had a series of studies in various mammalian species apparently established that amaranth was not embryotoxic in maternally tolerated doses than data from a study in the cat, a species not previously used in this context, renewed doubts about the colouring's safety with respect to gonadal and foetal development (Report of the Comptroller General of the United States Food and Drug Administration, MWD-76-40, 20 October 1975).

A further study was undertaken, therefore, in which groups of approximately 20 female cats were dosed daily for 6–16 days before pregnancy and then throughout the gestation period, with one, two or three gelatin capsules each containing 305 mg amaranth, giving a total daily dose of approximately 92, 187 or 264 mg/kg at the onset of pregnancy. The 43 controls were given three placebos each day. If oestrus did not occur naturally within 15 days of the start of amaranth treatment, it was induced by injection of pregnant-mare serum gonadotrophin, and ovulation was stimulated in all the cats by injection of human chorionic gonadotrophin before mating. Caesarean sections were performed on day 61 or 62 of gestation and all live foetuses were incubated for 24 hr to determine the survival rate, after which some foetuses from each litter were prepared for visceral examination and the rest were processed for determination of skeletal defects.

A red coloration of mucous membranes, skin, urine and faeces indicated that, in the cat, not all the amaranth administered was degraded to non-dye metabolites prior to distribution to the tissues. However, records of maternal body-weight gain, numbers of corpora lutea and incidences of pregnancy and abortion produced no evidence of any adverse effects related to the amaranth treatments. Data on total implantations, resorptions, dead foetuses, litter sizes, 24-hr postnatal viability, foetal weight, sex ratios of live foetuses and the incidence of foetal anomalies also showed no differences between control and treated animals indicative of any adverse effect by amaranth on the foetal development of the cat.

It was concluded, therefore, that results in this species supported those previously obtained in the mouse (Larsson, *Toxicology* 1975, **4,** 75), rat (Khera *et al. Fd Cosmet Toxicol.* 1974, **12,** 507; *Cited in F.C.T.* 1975, **13,** 473 & 1977, **15,** 153), rabbit (*ibid* 1975, **13,** 473) and dog (Mastalski *et al. Toxic. appl. Pharmac.* 1975, **33,** 122).

3195. Tobias acid eliminated unchanged

Marchisio, M. A., Dubini, M., Serra, G., Mennini, T. & Manara, L. (1976). Excretion of ^{35}S-Tobias acid (2-naphthylamino, 1-sulphonic acid) by the rat after oral and intravenous administration. *Br. J. ind. Med.* **33,** 269.

Tobias acid (2-naphthylamine-1-sulphonic acid), an important dyestuffs intermediate, is not a known carcinogen in animals or man. However it differs from the powerful carcinogen β-naphthylamine only in the presence of a sulphonic group ortho to the amino group, and an investigation of possible metabolic cleavage to free β-naphthylamine was considered desirable.

Six rats were given ^{35}S-labelled Tobias acid (1 mg/kg) by the oral or iv routes, and radioactivity in urine and faeces was determined for the next 3 days. After iv administration, most of the ^{35}S excreted within 72 hr appeared in the urine in the first 24 hr; after 72 hr 71–86% had been eliminated by this route. Small amounts (0·6–2·6%) were detected in the faeces. After oral administration prompt elimination again occurred, and by 72 hr 29–55% had appeared in the urine and 24–44% in the faeces, giving a total excretion of 69–85%. Chromatography of urine and faeces revealed a single peak of activity at the same R_F as Tobias acid, and there was no loss of urinary activity after addition of barium chloride, confirming the absence of significant amounts of free ^{35}S-labelled sulphate. As no attempt was made to wash the cages, it was assumed that the activity not recovered was trapped therein, although no analysis of body tissues was made.

The results indicate that Tobias acid is rapidly eliminated unchanged, without significant cleavage, conjugation or biotransformation. This is consistent with the finding that no metabolism takes place if the amino group of an arylaminosulphonic acid is hindered by an adjacent group or ring (McMahon & O'Reilly, *Fd Cosmet. Toxicol.* 1969, **7,** 497). Further investigation using ring-labelled Tobias acid of high specific activity would lower the limit of 2-naphthylamine detection and permit a more definite conclusion to be drawn.

3196. How the microflora deals with dyes

Larsen, J. C., Meyer, T. & Scheline, R. R. (1976). Reduction of sulphonated water-soluble azo dyes by caecal microorganisms from the rat. *Acta pharmac. tox.* **38,** 353.

The reduction of azo dyes such as amaranth by the gut microflora is well established (*Cited in F.C.T.* 1977, **15,** 77), and many nitro compounds may be reduced by the flora to more toxic metabolites (*ibid* 1977, **15,** 262).

In an attempt to bring *in vitro* conditions more into line with those found *in vivo*, whole-cell extracts of rat caecal contents were used, in preference to cell-free extracts, in studies of Fast Red E (CI no. 16045), Ponceau 4R (16255), Ponceau 6R (16290) and amaranth (16185). Each incubation was started with a concentration of 5×10^{-4} mmol of the colouring and the change in optical density was determined at 20-min intervals by spectrophotometry. The reduction of the substrate by the caecal extract started immediately, and Fast Red E was decolourized slightly faster than the other dyes. In contrast to earlier findings with cell-free extracts, there was no detectable increase in reduction rate with an increase in the number of sulphonate groups in the different dyes; the indication was that an increase in the degree of sulphonation slightly lowered the rate of reduction by caecal micro-organisms. Higher degrees of sulphonation and therefore increased ionization of the compound tend, however, to reduce the ability of the substrate to penetrate the cell wall, a fact that would explain the difference between these results and those obtained in experiments with cell-free extracts.

AGRICULTURAL CHEMICALS

3197. Inhaling azinphos-methyl

Kimmerle, G. (1976). Subchronic inhalation toxicity of azinphos-methyl in rats. *Arch. Tox.* **35**, 83.

The insecticide azinphos-methyl has an appreciable acute oral toxicity which is comparable with that of parathion. Prior to the investigation now reported, no repeated inhalation studies on the compound had been published and for this reason a TLV of 0·2 mg/m³ air was established in the USA only by analogy with other organophosphate pesticides.

Four groups of ten male and ten female rats were exposed in dynamic inhalation chambers to mean aerosol concentrations of azinphos-methyl in the air of 0, 0·195, 1·24 and 4·72 mg/m³ respectively. The controls inhaled only the solvent mixture of ethanol–polyethylene glycol 400, 1:1 v/v. The exposure periods were 6 hr/day, 5 day/wk for 12 wk. Animals were inspected daily and weighed weekly and the cholinesterase activity in plasma and erythrocytes was determined after 2, 4, 6, 8, 10 and 12 wk. Haematology, activity of the serum enzymes glutamic–oxalacetic transaminase, glutamic–pyruvic transaminase and alkaline phosphatase and the serum concentrations of urea, creatinine and bilirubin were examined after 12 wk of inhalation. At the end of the exposure period all animals were killed and gross and histological examinations of internal organs were carried out. Cholinesterase activity was also determined for the brain.

Exposure to the compound at concentrations up to 4·72 mg/m³ had no significant effect on appearance and behaviour, but male rats exposed to 4·72 mg/m³ showed a significantly lower body-weight gain. Haematological values, transaminase and alkaline-phosphatase activities and the serum concentrations of urea, creatinine and bilirubin were unchanged. Concentrations of 0·195 and 1·24 mg azinphos-methyl/m³ induced no depression of cholinesterase activity in plasma and erythrocytes but 4·72 mg/m³ inhibited activity by 30–40%. No changes were observed in the other parameters examined.

It appears from these studies that the present TLV of 0·2 mg/m³ includes an adequate safety factor for azinphos-methyl.

THE CHEMICAL ENVIRONMENT

3198. Lead and the placenta

Fahim, M. S., Fahim, Z. & Hall, D. G. (1976). Effects of subtoxic lead levels on pregnant women in the State of Missouri. *Res. Commun. chem. Path. Pharmac.* **13**, 309.

Lead is derived from a wide variety of environmental sources and may be of particular toxicological significance in young children (*Cited in F.C.T.* 1975, **13**, 277). It is known to cross the human placenta (*ibid* 1966, **4**, 533), and studies of lead in cord blood have been described (*ibid* 1975, **13**, 278). This study of the effect of subtoxic lead concentrations on the outcome of pregnancy adds to the picture.

The lead content of blood samples from the cord, placenta and placental membranes, as well as of maternal and foetal blood, from 249 women living in a non-mining area (A) was compared with that in samples from 253 women who were living in a lead-mining area (B) at the time of delivery. Pregnancies at term characterized by early membrane rupture constituted 0·41% of group A and 17% of group B, the one case in group A being in an insulin-dependent diabetic mother. The incidence of premature delivery in the two groups was 3 and 13·04%, respectively. There was a high correlation between maternal and foetal blood concentrations of lead, and both, like the lead levels of the membranes, were significantly higher in the pre-term pregnancies and those involving early membrane rupture than in the pregnancies of normal duration. The highest mean lead concentrations occurred in placental membranes, with levels of 38·9% μg/100 g in group A and 45 μg/100 g in group B. The concentration of lead in the membranes of term pregnancies with early membrane rupture was about six times greater than the placental level, and in premature pregnancies the increase was fourfold.

These data indicate that the incidence of early membrane rupture and premature delivery may be

raised by subtoxic levels of lead in the environment of the pregnant women. It is suggested that lead may decrease the collagen concentration of the membrane and cause it to rupture more readily. On the other hand, Meudt & Meudt (*Am. J. Obstet. Gynec.* 1967, **99,** 562) suggested that a decisive factor might be the extent of adherence between the amniotic sac and its surroundings.

3199. Lead uptake on low rations

Quarterman, J., Morrison, J. N. & Humphries, W. R. (1976). The effects of dietary lead content and food restriction on lead retention in rats. *Envir. Res.* **12,** 180.

Previous studies have shown that the levels of specific dietary components as well as the type of lead compound ingested may affect lead retention (*Cited in F.C.T.* 1976, **14,** 647). The size of a single dose of lead has been shown to have no effect on the proportion absorbed (*ibid* 1975, **13,** 478). In the experiments described below the effects on rats of reducing the total quantity of food available and of altering the concentration of lead in the diet are described.

Three different experimental regimes were used to compare the lead retention of animals on restricted diets with that of animals fed *ad lib.* The composition of the diet was the same for each experiment apart from the lead level. In experiment 1, young rats fed *ad lib.* with a diet containing 200 mg lead/kg for 38 days were compared with animals given 5 g of the same diet/day for an equal period. A similar comparison was made in experiments 2 and 3 but the restricted diets corresponded in quantity to the intake of pair-fed rats given a low-protein diet *ad lib.* A diet containing 400 mg lead/kg was fed for 23 days in experiment 2, and in experiment 3 a diet containing 200 mg lead/kg was given for 45 days. In all three regimes, the reduced diets increased the proportion of lead retained compared with that retained by controls fed *ad lib.*; growth rates were also reduced considerably. However, only in experiment 2, involving the higher lead level, did the reduced diet produce a significant increase in body levels of lead (μg lead/100 g body weight). Blood-lead levels were in all cases unaffected by the quantity of food available.

The authors point out that since most of the lead incorporated in the body is deposited in bone, it is possible that the reduced growth rate allows greater time for the incorporation of lead into the bones. However, they also cite experiments indicating that the influence of bone formation on lead retention is fairly weak (Quarterman *et al. Trace Substances Environ. Hlth* 1974, **7,** 347).

The effects on retention of different lead levels in the diet were studied separately. Groups of eight rats were given diets containing 0, 50, 200 or 400 mg lead/kg *ad lib.* for 3 wk and a fifth group was fed at the 200 mg level for 6 wk. The lead level in the diet did not affect the proportion of lead retained, a result in agreement with the previous findings with single doses mentioned above. The lead content of the carcasses was proportional to the dietary lead level and the duration of the experiment. Food intake decreased with increasing dietary lead level, which might have been expected to increase lead retention; the authors adjusted the retention figures on the basis of this expectation but still found no significant differences in lead retention.

Total food intake should be considered in any experiment concerned with the effects of dietary factors on lead retention. Food restriction may also aggravate the problem of lead poisoning in man, since poor urban children who tend to be the group most exposed to lead may also be poorly nourished.

3200. Nickel sensitization in the guinea-pig

Wahlberg, J. E. (1976). Sensitization and testing of guinea pigs with nickel sulfate. *Dermatologica* **152,** 321.

Although nickel is one of the common causes of contact dermatitis in man (*Cited in F.C.T.* 1976, **14,** 217), it has proved difficult to sensitize experimental animals to the element. Some success has followed the painting of guinea-pigs with nickel in sodium lauryl sulphate (Nilzén & Wikström, *Acta Derm.-vener., Stockh.* 1955, **35,** 292) and positive results have also been obtained in the guinea-pig maximization test (Magnusson & Kligman, In *Identification of Contact Allergens*; Thomas, Springfield, 1970).

Studies on the passive transfer of nickel allergy require highly sensitive guinea-pigs and a reliable test method suited to the special operating techniques used. In preparation for such a study, the investigation cited above compared two sensitization methods: the guinea-pig maximization (MK) test of Magnusson & Kligman (*loc. cit.*) and a combined injection and painting (PT) method devised by Polak & Turk (*Clin. exp. Immunol.* 1968, **3,** 245). Both were tested in female albino guinea-pigs of the Hartley strain. For the MK method, 1% nickel sulphate in Freund's adjuvant was used for intradermal injection and 5% nickel sulphate in petrolatum for topical application. For the PT method, a single dose of 1 ml 1% nickel sulphate in complete Freund's adjuvant was divided into 0·2 ml amounts and injected at five different intramuscular sites (total 10 mg of the salt). This was followed after 2 wk by 0·1 ml of 0·25% nickel sulphate in 0·15 M-NaCl injected intracutaneously, a procedure repeated four times at 1-wk intervals; at the same time, painting was begun with 5% nickel sulphate in 1% Triton X-100 or in 1% sodium lauryl sulphate and repeated four times at intervals of 1 wk. Following this, the first test after the start of sensitization was performed. A second series of treatments was then undertaken to investigate whether a higher degree of sensitivity could be obtained. In the test method, 0·1 ml nickel sulphate in a concentration of 0·5, 0·25, 0·125 or 0·0625% was injected intradermally into the shaven flanks of guinea-pigs.

Because nickel causes reactions in normal (unsensitized) animals, it is difficult to decide whether a sensitizing treatment has been effective, and control readings in normal animals are therefore used for comparison. The intradermal test method with nickel in four different concentrations yielded closely similar results in six series of tests in normal animals, and its reproducibility was considered satisfactory for comparing the different methods of sensitization in

the induction of nickel allergy. Stronger reactions were obtained with increasing nickel concentration. It was observed that pretested guinea-pigs proved more difficult to sensitize and gave rise to weaker reactions, features that the author attributes to the development of tolerance. The PT method resulted in a slightly higher sensitivity with Triton X-100 than with sodium lauryl sulphate as the vehicle for topical application of nickel. In the comparison between MK and PT methods, no differences in effectiveness were observed unless the animals had been pretested, in which case the PT method was the more effective.

The author recommends an examination of methods for increasing the sensitivity still further although he recognizes difficulties in increasing the concentration of nickel in Freund's adjuvant at the first injection in the PT method, since some deaths from nickel poisoning were encountered with the dose used.

3201. Using nitroprusside safely

Vesey, C. J., Cole, P. V. & Simpson, P. J. (1976). Cyanide and thiocyanate concentrations following sodium nitroprusside infusion in man. *Br. J. Anaesth.* **48**, 651.

Patients given iv infusions of sodium nitroprusside (SNP) to control blood pressure during surgery have shown a four-fold increase in plasma cyanide concentration but only a slight increase in plasma thiocyanate, despite the fact that thiocyanate is the major metabolite of cyanide in the body.

In 26 patients undergoing major orthopaedic surgery, short periods of SNP infusion produced a considerable increase in cyanide, both in red cells and in plasma, both levels being linearly related to the total dose of SNP. Plasma changes in cyanide were reflected in changes of cyanide in expired air. Shortly after the SNP infusion had been completed 98·4% of the cyanide in the blood was present in the red cells, and both mean red cell and plasma cyanide concentrations fell to less than half their immediate post-infusion levels within 1 hr. In contrast, changes observed in plasma thiocyanate were small, and varied widely in relation to the total dose of SNP used. Thus the rate of cyanide detoxication was slower than that of release of cyanide from SNP.

In view of the risk of producing toxic plasma concentrations of cyanide, the authors suggest that the total dose of SNP infused should be limited during an average surgical operation to 1·5 mg/kg body weight. In situations where this dose is likely to be exceeded, it is considered advisable to monitor the plasma cyanide concentration, so that administration of SNP can be discontinued before a toxic accumulation of cyanide results.

3202. The industrial hazards of chloroform

Torkelson, T. R., Oyen, F. & Rowe, V. K. (1976). The toxicity of chloroform as determined by single and repeated exposure of laboratory animals. *Am. ind. Hyg. Ass. J.* **37**, 697.

High oral doses of chloroform have recently been reported to produce kidney tumours in male rats and liver tumours in mice of both sexes, although female mice were the more susceptible (Federal Register, 1976, **41**, 15026). In mice, as in rats, the males were more sensitive to the nephrotoxic effects of chloroform, a finding that correlated with the much greater quantities of radioactivity bound to the male renal cortex and medulla after administration of ^{14}C-labelled chloroform (*Cited in F.C.T.* 1976, **14**, 60). In pregnant rats, chloroform inhaled at concentrations of 30–300 ppm for 11 days produced a decrease in maternal food consumption and weight gain, accompanied by retarded foetal development and decreased foetal body measurements, even at the lowest level, but gross liver changes were seen only at 300 ppm (*ibid* 1975, **13**, 402). Longer-term inhalation studies and acute tests by other routes, reported briefly in 1965 (American Industrial Hygiene Association, *Am. ind. Hyg. Ass. J.* 1965, **26**, 636), are now reported in full for the first time.

In male rats an oral LD_{50} value of 2·0 g/kg was obtained for undiluted chloroform, but liver and kidney damage was evident even from a single dose of 0·25 g/kg. On the uncovered ear of rabbits, up to ten applications produced slight hyperaemia and exfoliation, and one or two 24-hr covered applications to the shaved rabbit belly resulted in hyperaemia, necrosis, scab formation and delayed healing. Single 24-hr applications of 1·0–3·98 g/kg under a plastics cover to the rabbit belly produced extensive necrosis and weight loss, and autopsy after 2 wk revealed degeneration of the kidney tubules, although the livers were not grossly affected. In the rabbit eye, chloroform caused slight conjunctival irritation and corneal injury, which was not reduced in severity by washing the eye 30 sec later.

When rats were exposed for 7-hr periods to atmospheric concentrations of 25, 50 or 85 ppm (120–410 mg/m^3), on 138–144 days out of a total period of 195–203 days, males at all levels showed an increase in relative kidney weights, and histological examination revealed cloudy swelling of the renal tubular epithelium and centrilobular granular degeneration with focal areas of necrosis throughout the liver. In addition there was a decrease in body weight at 50 and 85 ppm, and an increase in relative liver weight and pneumonia at the highest level. Females at the lowest level showed only an increase in relative kidney weights, but at the two higher levels liver and kidney pathology was similar to that in the males. Male rats allowed to recover for 6 wk after their last exposure to 25 ppm were normal in all respects, as were male rats exposed for 1, 2 or 4 hr daily to 25 ppm. Even at the highest exposure level, terminal blood counts and urine analyses were unaffected and serum levels of urea nitrogen, glutamic–pyruvic transaminase and alkaline phosphatase were within normal limits, despite the liver damage observed histologically. Dogs exposed to 25 ppm and rabbits and guinea-pigs exposed to 25, 50 or 85 ppm under the same conditions as the rats showed somewhat inconsistent liver, kidney and lung changes. The fact that these were most obvious at 25 ppm may have been due to the small number of survivors at higher levels, a situation not considered to be related to treatment since high death rates also occurred in the air-exposed controls.

On the basis of these findings it was recommended that workers should avoid eye contact or prolonged skin contact with chloroform, and that the time-weighted average concentration in industrial atmospheres should not exceed 10 ppm, with a 25 ppm ceiling for repeated exposures.

[The American Conference of Governmental Industrial Hygienists recently proposed to lower the TLV for chloroform from 25 to 10 ppm, but on grounds of suspected carcinogenicity rather than of systemic toxicity (TLVs. Threshold Limit Values for Chemical Substances and Physical Agents in the Workroom Environment with Intended Changes for 1976, American Conference of Governmental Industrial Hygienists, P.O. Box 1937, Cincinnati, Ohio 45201; pp. 94, $1.00). As the studies described above were of only about 6 months' duration, they were insufficient for an assessment of carcinogenicity.]

3203. A long breath of cumene

Seńczuk, W. & Litewka, B. (1976). Absorption of cumene through the respiratory tract and excretion of dimethylphenylcarbinol in urine. *Br. J. ind. Med.* **33,** 100.

Animal studies have shown that cumene (isopropylbenzene) is a relatively innocuous hydrocarbon when inhaled (*Cited in F.C.T.* 1971, **9,** 598).

In the studies described in the paper cited above, ten healthy people were exposed in an inhalation chamber to various concentrations of cumene over a simulated working day of 8 hr; two periods of 2·5 hr, each followed by a break of 30 min, with a final 2-hr exposure. The subjects inhaled air containing 240, 480 or 720 mg cumene/m^3 with a 10-day interval between successive exposures. Cumene was determined in expired air during the initial and final phases of exposure, and the main urinary metabolite, dimethylphenylcarbinol, was determined in samples collected during exposure and for up to 48 hr after its commencement.

Retention in the respiratory tract ranged from 64 to 45% of the inhaled cumene, depending on the time of exposure. The amount of cumene absorbed was directly proportional to the urinary excretion of its metabolite. Retention of cumene tended to diminish towards the end of each period of exposure. Urinary excretion of dimethylphenylcarbinol approached zero 48 hr after the start of exposure, and the total excretion of metabolite in the urine served as an index of the degree of exposure to cumene during an 8-hr working day.

3204. Lighting up polytetrafluoroethylene

Lee, K. P., Zapp, J. A., Jr. & Sarver, J. W. (1976). Ultrastructural alterations of rat lung exposed to pyrolysis products of polytetrafluoroethylene (PTFE, Teflon). *Lab. Invest.* **35,** 152.

Toxic breakdown products of polytetrafluoroethylene (PTFE) take on significance only at temperatures exceeding those achieved in ordinary cooking procedures (*Cited in F.C.T.* 1974, **12,** 794). The finding that more fluoride ion, derived it is thought from carbonyl fluoride, is excreted in the urine of persons who suffer from polymer fume fever (*ibid* 1975, **13,** 590) has focused attention upon the gaseous products of pyrolysis, but it now seems that the particulate products may also be of toxicological significance.

Rats were exposed to the fumes of PTFE heated at 380, 400, 425 or 450°C for 4 hr. Fumes from pyrolysis at temperatures below about 425°C produced no clinical signs or lung alterations, and at such temperatures the pyrolysis products yielded no particles when passed through a Millipore filter of pore size 0·2 μm. At 450°C many particles of diameter 0·02–0·04 μm were collected in the filter, while pyrolysis of PTFE at 550°C produced, in addition, small numbers of large spherical particles measuring 0·7–5·0 μm. Rats exposed to pyrolysate produced at 450°C showed respiratory difficulty and five, seven and eight of eight rats died in three separate exposure trials, pulmonary oedema and haemorrhage as well as necrosis of the tracheobronchial epithelium being evident at autopsy. Electron microscopy showed cytoplasmic swelling, bleb formation, fragmentation and denudation of the membranous pneumocytes, and to a lesser extent of the endothelial cells of the lung. In rats exposed to pyrolysates filtered to exclude the small particles, the only signs of toxicity were minor degenerative changes in the tracheobronchial epithelium. From these results and the size distribution of the particles produced from PTFE at 450°C, the particles 0·02–0·04 μm in diameter appear to be the toxic component responsible for the pulmonary oedema and associated lung reactions.

3205. Inhaling cyclopentenone

Kruysse, A. & Feron, V. J. (1976). Repeated exposure to cyclopentenone vapour: Long-term study in Syrian golden hamsters. *Zentbl. Bakt. Hyg.* IB, **163,** 448.

Cyclopentenone occurs in cigarette smoke and wood smoke, and some knowledge of its toxicity and carcinogenicity following inhalation is therefore desirable. In preliminary studies, a 4-hr LC_{50} value of 640 ppm (2150 mg/m^3) was obtained in hamsters, and twice-weekly intratracheal instillation of a saline solution of cyclopentenone into hamsters for 12 months produced tracheobronchitis but no evidence of carcinogenicity.

In the present study hamsters were exposed for 7 hr daily on 5 days/wk to an atmospheric concentration of 18 ppm (60 mg/m^3) for 52 wk, and subsequently to 27 ppm for 26 wk. A further group was exposed to 18 ppm for 52 wk and given fortnightly intratracheal instillations of 0·5% benzo[a]pyrene or 0·125% diethylnitrosamine in saline or of saline alone. Exposure to cyclopentenone did not affect behaviour, condition or death rate, compared with that in air-exposed controls, but produced a significant depression in body weight in both sexes and a slight increase in the relative liver weight of females. The only histopathological finding of note was renal amyloidosis, the incidence of which was slightly greater in females exposed to cyclopentenone and treated with benzo[a]pyrene, diethylnitrosamine or saline than in those exposed to air and treated similarly. No evidence of carcinogenicity or of co-carcinogenicity was found, however.

3206. Hexane straining every nerve

Schaumberg, H. H. & Spencer, P. S. (1976). Degeneration in central and peripheral nervous systems produced by pure *n*-hexane: An experimental study. *Brain* **99**, 183.

Paulson. G. W. & Waylonis. G. W. (1976). Polyneuropathy due to *n*-hexane. *Archs intern. Med.* **136**, 880.

Hexane has been subjected to an increasingly bad press over the past decade. Its neurotoxic activity, featured in isolated case histories of morbid glue-sniffers (*Cited in F.C.T.* 1976, **14**, 157), has also been evident in the industrial context, with a number of individuals developing polyneuropathy when exposed to high atmospheric concentrations of the solvent (*ibid* 1973, **11**, 157). In the two studies cited here, hexane neutrotoxicity has been investigated further, in the rat and man.

In the first study, a group of eight rats was exposed continuously to an atmosphere containing 400–600 ppm hexane for up to 162 days. An unsteady gait developed in the animals from day 45, further exposure producing distal hindlimb weakness with footdrop. By contrast, there was no evidence of clinical neuropathy in three rats given hexane by sc injection (on 5 days/wk for up to 35 wk, to provide total doses of 98·7, 102·8 and 198 g/kg, respectively).

Nevertheless pathological changes occurred in both groups of animals. In two of the animals given hexane by injection and in the four rats exposed to the vapour for more than 42 days, there was widespread degeneration of myelinated fibres in the central and peripheral nervous systems, most prominently in the tibial nerves supplying the calf muscles. Local axonal dilatation and localized fibre swelling were the first detectable lesions, the axonal swellings, surrounded by abnormally thin myelin sheaths, being tapered and discontinuous. Large numbers of neurofilaments (about 10 nm) were associated with the swollen regions of the central and peripheral myelinated axons and there was an abnormally high frequency of Schwann cells in adjacent areas.

Giant axonal swellings have previously been observed in nerve biopsies of subjects inhaling glue vapours (*ibid* 1976, **14**, 157) but not in the peripheral nerves of animals experimentally exposed to hexane. However, widespread axonal degeneration in the central nervous system was among the effects induced with methyl *n*-butyl ketone (MBK) in rats (*ibid* 1976, **14**, 157) and it has been demonstrated also in *n*-hexane-induced neuropathy in mice. The authors speculate that similar lesions are likely to occur in human neuropathies caused by *n*-hexane and other toxic chemicals but may be masked by the accompanying dysfunction of peripheral nerves. While the latter effect may regress on termination of exposure because of the capacity of peripheral nerves to regenerate, it is considered that the central nervous system would be unlikely to recover to a similar degree and lesions in this region could therefore be responsible for permanent sensory loss, ataxia or spasticity.

Cases of polyneuropathy in a printing works are described in the second paper cited above. Over a 25-yr period, four distinct episodes occurred involving eight of the 50 employees altogether. All those affected worked in the printing room. In the latest instance, reported in 1973, one of the two men concerned suffered from leg weakness, and electromyograms indicated the presence of neuropathic and myopathic disorders together with peripheral neuropathy. Generally, conduction velocities in the motor nerves and serum-cholinesterase activity were markedly reduced in the patients, the latter finding contrasting with the elevated serum-cholinesterase activities observed in cases of neuropathy resulting from MBK exposure. Recovery was complete in most cases within 6 months. Air analyses at the plant showed that hexane was the only material present above its TLV, typical levels being 2000–4000 mg/m^3 (TLV 1800 mg/m^3, 500 ppm).

[The animal study demonstrating serious neural complaints after relatively short, although admittedly continuous, exposure to hexane in concentrations around the TLV suggests again that a re-examination of the TLV might be advisable.]

3207. A firm case against MBK

Spencer, P. S. & Schaumburg, H. H. (1976). Feline nervous system response to chronic intoxication with commercial grades of methyl *n*-butyl ketone, methyl *iso*butyl ketone, and methyl ethyl ketone. *Toxic. appl. Pharmac.* **37**, 301.

Neuropathy has been reported among spray painters and other workers using solvents containing methyl *n*-butyl ketone (MBK) mixed with other ketones (*Cited in F.C.T.* 1977, **15**, 159). Animal studies have confirmed that MBK causes neuropathy (*ibid* 1976, **14**, 157). The possible role of the diluent, methyl ethyl ketone (MEK), in producing this neuropathy has given rise to other studies (*ibid* 1977, **15**, 256), and this aspect of the problem and the possible neurotoxicity of the isomeric methyl isobutyl ketone (MIBK) have now been investigated in cats.

Cats were given the test compounds in twice-daily sc injections of 150 mg/kg. Treatments, with commercial MBK (containing 2·899% MIBK and traces of mesityl oxide and unidentified contaminants), with commercial MEK (containing traces of acetone, *sec*-butanol and unidentified compounds), with commercial MIBK (containing 0·94% MBK and traces of acetone and other compounds) or with 9:1 mixtures of MEK with either MBK or MIBK, were continued on 5 days/wk for up to 8·5 months. Biopsy specimens from the central plantar toepads, branches of the lateral plantar nerve and portions of a superficial interosseous muscle were taken at 45 and 135 days for examination by light and electron microscopy. More extensive tissue samples from cats treated for 2, 4 or 6 months were examined *post mortem* after systemic perfusion with fixatives.

The animals varied considerably in their ability to tolerate the ketone injections and responses ranged from narcosis and salivation to generalized weakness and eventual death in a few animals. Neurological dysfunction appeared only in those cats given MBK alone. These developed peripheral neuropathy with hindquarter weakness at 8–10 wk, and severe hindlimb footdrop after 10–12 wk; after 16 wk they were

unable to walk. Microscopy of nerve fibres showed lesions only after exposure to MBK alone or to the MEK–MBK mixture. Early lesions were apparent in plantar nerves and some pacinian corpuscles from animals exposed to MBK on 45 days, and the proliferation of axonal neurofilaments in pacinian corpuscles resembled that seen during experimental acrylamide neuropathy. Hindfoot tissue from cats treated with an MEK–MBK (9:1) mixture showed no changes after 45 or 135 days of intoxication. Tibial nerve branches supplying calf muscles and sampled after 8·5 months of intoxication contained abnormal numbers of fibres displaying segmental remyelination. Perfused tissues from cats treated with MBK showed prominent giant axonal swelling of myelinated fibres, enlargement of nerve terminals and total fibre breakdown in the distal region of long ascending and descending pathways in the spinal cord and medulla, and in preterminal and terminal axons in grey matter adjacent to the aqueduct.

3208. A breath of isocyanate

Axford, A. T., McKerrow, C. B., Parry Jones, A. & Le Quesne, P. M. (1976). Accidental exposure to isocyanate fumes in a group of firemen. *Br. J. ind. Med.* **33**, 65.

Le Quesne, P. M., Axford, A. T., McKerrow, C. B. & Parry Jones, A. (1976). Neurological complications after a single severe exposure to toluene diisocyanate. *Br. J. ind. Med.* **33**, 72.

Gardiner, J. S. (1976). Chemicals in the fire. *Lancet* **II**, 365.

Toluene diisocyanate (TDI) is a severe irritant and has been reported to induce inhalational allergic pneumonitis (*Cited in F.C.T.* 1977, **15**, 255). In polyurethane workers, TDI has been held responsible for sensitization leading to deterioration of ventilatory capacity and persistent respiratory-tract symptoms (*ibid* 1976, **14**, 218).

The first paper cited above describes the effects of exposure to TDI and possibly other fumes on 35 firemen fighting a fire in a polyurethane foam factory. During the course of the fire, and during the following 3 wk, most of the exposed men suffered from gastrointestinal, respiratory or neurological symptoms. Nausea affected 15 men and was complicated by vomiting in three and by abdominal pain in four. These effects subsided within 2 days and left no residual complaints. Tightness of the chest, breathlessness and cough occurred in 14 men while they were at the scene of the fire, and in 17 others more than 8 hr afterwards. Respiratory symptoms were most pronounced within the first 3 days and generally improved thereafter; but a review 6 months later indicated that some men had sustained long-term respiratory-tract damage involving a decline in ventilatory capacity. These men claimed an increased susceptibility to upper-respiratory-tract infections and four bronchitis sufferers felt that their symptoms had increased. In 20 of the men, some respiratory effects persisted nearly 4 yr later. Six of these men were classed as simple bronchitis sufferers and six as chronic bronchitis sufferers.

The evidence of long-term respiratory damage caused by TDI is inconclusive, particularly as a correlation between loss of pulmonary function and exposure to fires has been observed in other fire-fighters. However, in general, the fireman in this study showed a marked decline in ventilatory capacity during the 6 months after the fire, with subsequent recovery rather than a sustained decline.

The second paper describes neurological signs in 23 of these firemen. Five men were affected during the fire with symptoms including euphoria, ataxia and loss of consciousness. In these five and in nine others, various combinations of headache, difficulty in concentration, memory defects and confusion developed during the next 3 wk. When 18 men were examined again 4 yr later, 13 (including eight who had not reported neurological disturbances after 3 wk) complained of some combination of poor memory, personality changes, irritability or depression. In those whose neurological symptoms persisted after 4 yr, a selective defect of long-term memory recall was detected. Although there is no proof that TDI was responsible for these effects, the men concerned were exposed to large quantities of TDI.

The author of our third citation observes the difficulty in a complicated major fire of identifying with certainty the cause or causes of adverse effects. TDI has previously been observed to affect respiration, and it is thus a very likely cause of the respiratory difficulties described above. However, neurological disturbances due to TDI have not been observed previously and its implication in these effects is more doubtful.

NATURAL PRODUCTS

3209. Medicinal garlic and onions

Sainani, G. S., Desai, D. B. & More, K. N. (1976). Onion, garlic, and atherosclerosis. *Lancet* **II**, 575.

The recent claims that onion and garlic may have inhibitory effects on atherosclerosis have been based on acute clinical studies using volunteers, or on experiments with rabbits (*Cited in F.C.T.* 1976, **14**, 651). An epidemiological study has now been reported.

Healthy subjects of the Jain community of India were divided into three groups on the basis of their onion and garlic consumption. A group of 45 high consumers on a mixed but mainly vegetarian diet and a group of 35 people who ate small amounts of onion and garlic in a strict vegetarian diet were compared with a control group of 33 "orthodox Jains", strict vegetarians who had never eaten onion or garlic. The three groups were all closely matched for age, sex and social class.

There were no significant differences in serum cholesterol or coagulation times between the three groups. However orthodox Jains had significantly higher ($P < 0·01$) phospholipid and plasma-fibrinogen

levels than the other two groups and the highest onion and garlic eaters had decreased serum-triglyceride ($P < 0.01$) and β-lipoprotein levels ($P < 0.05$). It was concluded that the regular consumption of onion and garlic had "a protective effect on some important factors which influence atherosclerosis".

[Before garlic and onion become accepted in a protective role, more conclusive epidemiological evidence is needed, this particular study being open to obvious criticism over group sizes. Studies relating garlic/onion intake to actual incidences of atherosclerosis rather than to contributory factors might be more valuable.]

3210. The biochemical basis of ackee poisoning

Tanaka, K., Kean, E. A. & Johnson, B. (1976). Jamaican vomiting sickness. Biochemical investigation of two cases. *New Engl. J. Med.* **295**, 461.

Jamaican vomiting sickness, characterized by severe vomiting, is linked with the ingestion of unripe ackee. The toxic principle in the unripe fruit is hypoglycin A, which is metabolized in rats to methylenecyclopropylacetic acid (MA); this suppresses acyl coenzyme A dehydrogenase activity and thus inhibits trans-mitochondrial transport of long-chain fatty acids, which in turn leads to the depression of gluconeogenesis (*Cited in F.C.T.* 1967, **5**, 839; *ibid* 1972, **10**, 277). Rats treated with hypoglycin A have been shown to excrete massive amounts of dicarboxylic acids with 5–10 carbons atoms (Tanaka, *J. biol. Chem.* 1972, **247**, 7465), and to have high serum levels of isovaleric and α-methylbutyric acids (Tanaka *et al. Science, N.Y.* 1972, **175**, 69). However, none of these abnormal acids has before now been identified in the body fluids of humans suffering from the disease.

The paper cited above now reports that analysis of urine from two young Jamaican children who were victims of vomiting sickness revealed MA levels of 8 and 16 μg/mg creatinine, respectively, whereas none was found in the urine of five normal American children. The urine of the victims also contained high levels of adipate, glutarate, 2-ethylmalonate, suberate (octanedioate), 4-decenedioate, sebacate (decanedioate) and 2-methylsuccinate, all of which except 2-ethylmalonate had previously been detected in the urine of hypoglycin-treated rats. By contrast, in the urine of four normal children only 2-ethylmalonate and 2-methylsuccinate were present at detectable levels, and it was estimated that excretion of the dicarboxylic acids was increased by some 70–1000 times in vomiting sickness. Large amounts of β-hydroxyisovalerate, β-hydroxyisobutyrate and *n*-hexanoylglycine, and smaller quantities of isovaleryl- and *n*-butyrylglycines, were also found in the urine of the Jamaican victims, although the acylglycines were present at much lower levels than in the urine of hypoglycin-treated rats. Excretion of both free and conjugated forms of *n*-hexanoic, *n*-butyric, isobutyric, propionic, isovaleric, crotonic and β-methylcrotonic acids (the last two perhaps derived from β-hydroxybutyric and β-hydroxyisovaleric acids during analysis) was increased by up to 300 times, and serum levels of these short-chain fatty acids were increased by up to 23 times. No abnormal metabolites could be detected in the urine of six normal Jamaican adults who had eaten ripe ackee during the previous week, or in that of five American patients with Reye's syndrome, which has clinical manifestations similar to those of vomiting sickness.

The intake of hypoglycin in the form of unripe ackee is clearly related to the origin of Jamaican vomiting sickness. The findings also indicate that the modes of action of hypoglycin A in humans and rats are identical, although the alternative metabolism of isovalerate and butyrate appeared to be different in the two species. In the human subjects, isovaleric acid seemed to be metabolized mainly to methylsuccinic and β-hydroxyisovaleric acids, and butyric acid was excreted largely as ethylmalonic acid, whereas in the rat the glycine conjugates of the parent acids were produced in large amounts.

3211. A diet of rapeseed

Sharpe, G. L., Larsson, K. S. & Liedén, S. Å. (1975). Toxicological and teratological studies of a rapeseed protein diet in rats and mice. *Nutr. Metabol.* **18**, 245.

Loew, F. M., Doige, C. E., Manns, J. G., Searcy, G. P., Bell, J. M. & Jones, J. D. (1976). Evaluation of dietary rapeseed protein concentrate flours in rats and dogs. *Toxic. appl. Pharmac.* **35**, 257.

Fatty deposition in the heart muscle of animals given a diet in which rapeseed oil is a major energy source has been widely attributed to the erucic acid content of the oil (*Cited in F.C.T.* 1977, **15**, 348), while hydrolysis products of glucosinolates have been implicated in the toxic effects associated with some rapeseed products. Furthermore, rapeseed flour may contain antithyroid substances, which are widespread in plants of the Brassica genus (Golberg, *J. R. Coll. Physcns Lond.* 1967, **1**, 385). The safe use of such products for protein supplementation depends upon eliminating or reducing the contents of these toxic principles.

Pregnant rats fed rapeseed protein containing 0·2 mg glucosinolates/g for the first 18 days of gestation showed no teratogenic effects, but after delivery anorexia and weight loss were apparent. In most of them the fur was stained with a reddish discharge (not blood) from the nostrils. (A similar discharge developed in control rats fasted after day 18.) The treated dams were apathetic, failed to clean their pups and instead showed an increased tendency to eat them. Administration of rapeseed protein by gavage during the anorectic period produced profound lethargy, and profuse nasal and lachrymal discharge on day 18 or later. These reactions were unaffected by vitamin supplements. When the rapeseed-protein diet was given for 3–6 wk before mating, no toxic effects were observed until day 18 of gestation. Rats on a casein reference diet, given glucosinolate by gavage or mixed with the diet at levels up to 0·2 mg/g pure protein, showed no toxic response, possibly because this diet contained no myrosinase, on the presence of which the ultimate toxicity of glucosinolates to rats has previously been shown to depend. No ill effects were observed in the offspring of rats fed rapeseed protein, and the effects on pregnant rats suggest an

interaction with increasing endogenous levels of oestrogen and progesterone. In pregnant mice receiving the rapeseed-protein diet or a casein reference diet *ad lib.*, no toxic signs such as anorexia, weight loss or nasal discharge were present at any time.

Antithyroid activity of two protein-rich rapeseed flours with a low glucosinolate content was studied in the second paper cited above. Semi-synthetic diets providing 20 or 40% of total protein as rapeseed flour, with the remainder as casein, were fed to growing dogs and rats for 90 days. The flours contained oxazolidinethione (goitrin). which apparently retards the incorporation of iodine into thyroid hormones, but even with the higher goitrin content (0·287 mg/g flour), antithyroid effects were low, uptake of iodine-131, serum-thyroxine concentration, thyroid weight and histology being within normal limits. In the rats, the activity of serum glutamic–pyruvic transaminase and the mean corpuscular haemoglobin concentration fell and the packed cell volume and red cell count rose in proportion to the rapeseed-protein level in the diet, but these changes did not appear in the dogs. The authors conclude that the flours used in these studies were less toxic than previously tested flours of this type.

3212. Soy sauce: an irritating elixir

MacDonald, W. C. & Dueck, J. W. (1976). Long-term effect of shoyu (Japanese soy sauce) on the gastric mucosa of the rat. *J. natn. Cancer Inst.* **56**, 1143.

Epidemiological studies have suggested that dietary factors might be responsible for the high incidence of gastritis and mortality from gastric cancer in Japan. Vegetables pickled in shoyu, a sauce that figures prominently in traditional Japanese recipes, have previously been shown to cause reversible injury to the human gastric mucosa (MacDonald *et al. Can. med. Ass. J.* 1967, **96**, 1521). Shoyu is prepared from wheat and soya bean fermented in brine, fungi being used to start the fermentation process; it may injure the gastric mucosa because of its high salt content and may also contain unidentified mycotoxins. The long-term effects of commercial shoyu on the rat have now been reported.

Two groups of 24 female rats received from the age of 4 months a standard diet or a standard diet mixed with 50 ml shoyu/100 g meal. Two further groups of 21 and 20 animals underwent fundusectomy, which has been reported to increase susceptibility to gastric cancer, and were then maintained on analogous dietary regimes. All groups were observed from month 12 to 29, after which the animals, then aged 33 months, were killed.

The rats receiving a shoyu-treated diet were smaller, lived longer and were more active and apparently healthier than the control animals. Tumour incidences in the shoyu-fed rats, both fundusectomized and intact, were not statistically different from those in controls except in the case of mammary tumours, which developed in ten control rats but in none given shoyu. The frequency of mammary tumours appeared to be correlated with body weight. The consumption of shoyu-treated food over this prolonged period was associated with mucosal loss and nuclear changes in the surface epithelium of the gastric mucosa in some rats and occasionally with a mild gastritis, but provided no evidence of carcinogenicity.

3213. Teratology of zearalenone

Ruddick, J. A., Scott, P. M. & Harwig, J. (1976). Teratological evaluation of zearalenone administered orally to the rat. *Bull. env. contam. & Toxicol.* (U.S.) **15**, 678.

Zearalenone (F-2 toxin) is an oestrogenic mycotoxin produced by several species of Fusarium, a genus associated with a growing number of mycotoxins (*Cited in F.C.T.* 1974, **12**, 287).

Female rats were given 1, 5 or 10 mg zearalenone/kg/day by stomach intubation from day 6 to day 15 of gestation, and were examined on day 22. No signs of any maternal toxicity appeared, and no lesions of maternal viscera were seen. In the highest dose group, maternal weight gain and mean foetal weight were significantly below control values, and there was a slight but not significant increase in the incidence of embryonic deaths in this group. Skeletal defects, mainly affecting the ribs, sternum and tarsal and parietal bones, occurred in 12·8% of foetuses in the 1-mg/kg group, in 26·1% in the 5-mg/kg group and in 36·8% in the 10-mg/kg group. In the high-dose group the most prominent finding was arrested parietal ossification. This osteogenic effect of zearalenone has previously been reported in connexion with the 'splayleg' condition found in pigs (Miller *et al. Vet. Rec.* 1973, **93**, 555).

3214. Teratogenicity of patulin and its cysteine adducts

Ciegler, A., Beckwith, A. C. & Jackson, L. K. (1976). Teratogenicity of patulin and patulin adducts formed with cysteine. *Appl. envir. Microbiol.* **31**, 664.

Patulin, a toxic and carcinogenic heterocyclic lactone produced by a variety of fungi liable to occur in food and feedstuffs, has been identified in rotten apples and cider products. It has been reported to inhibit lactic dehydrogenase, alcohol dehydrogenase and muscle aldolase, the inhibition of the first and last of these enzymes being reversed or reduced by cysteine, with which patulin forms an adduct (Ashoor & Chu, *Fd Cosmet. Toxicol.* 1973, **11**, 617 & 995).

Aqueous solutions of patulin and of patulin–cysteine (1:1 and 1:2) adducts were injected into the air sac of fresh fertile hens' eggs and eggs containing 4-day-old embryos. On day 20 of incubation, embryos were examined for gross teratological effects. The LD_{50} of patulin was 68·7 μg for embryos injected before incubation and 2·35 μg for 4-day embryos, with a broad range of values. Embryos treated with 10 μg patulin before incubation or with 1–2 μg at the 4-day stage showed teratogenic changes, which were more marked in the latter group and included a predominance of splayed foot and malrotated ankle.

The acute toxicity of patulin was greatly reduced by reaction with cysteine, a dose of the lactone approximately 50 times the LD_{50} causing no deaths in 4-day embryos when it was injected as the cysteine adduct. However, 12% of the 4-day embryos treated

with 15 μg patulin as the adduct and 20% of those treated with 150 μg developed defects, primarily affecting the foot. Thus adducts of patulin with cysteine, although far less acutely toxic than the free lactone, remain teratogenic.

It is pointed out that the adducts are insoluble in the organic solvents used to extract patulin and would therefore not be detected in food or feedstuffs by the usual assay procedures.

COSMETICS, TOILETRIES AND HOUSEHOLD PRODUCTS

3215. A clean bill for NTA mutagenicity

Kramers, P. G. N. (1976). Mutagenicity studies with nitrilotriacetic acid (NTA) and Citrex S-5 in Drosophila. *Mutation Res.* **40**, 277.

Nitrilotriacetic acid (NTA) is the chelating agent that was introduced as a possible substitute for sodium triphosphate in household detergents to help counteract the havoc caused by excess phosphates in environmental waters. However, NTA itself has not been entirely free from possible problems. Results of carcinogenicity studies provided no clear evidence of any tumorigenic effects (*Cited in F.C.T.* 1975, **13**, 409), but it was considered that by increasing the transmission of heavy metals across the placental barrier NTA might give rise to possible teratogenic effects, although again any direct support for this contention was lacking (*ibid* 1973, **11**, 674; *ibid* 1974, **12**, 421). Citrex S-5, a mixture of sodium salts of sulphopolycarboxylic acids, has also been considered as a possible substitute for phosphates.

Studies in *Drosophila melanogaster* have given no indication that either NTA or Citrex S-5 can induce sex-linked recessive lethal mutations. Although an initial experiment suggested that NTA might increase mutation frequency, repetition failed to produce any more evidence of this. In the dominant lethal tests, no decrease in egg hatchability was observed after treatment of the flies with either compound. The studies provided no evidence that NTA or Citrex S-5 presented a genetic hazard.

3216. Profile of bronidox, a preservative for cosmetics

Potokar, M., Greb, W., Ippen, H., Maibach, H. I., Schulz. K. H., Lorenz, P. u. Gloxhuber, C. (1976). Bronidox, ein neues Konservierungsmittel für die Kosmetik: Eigenschaften und toxikologisch-dermatologische Prüfergebnisse. *Fette Seifen AnstrMittel* **78**, 269.

Bronidox, 5-bromo-5-nitro-1,3-dioxan, is an antimicrobial preservative intended for inclusion in cosmetic formulations.

The acute LD_{50} of Bronidox was 590 mg/kg orally in mice, and 455 mg/kg orally and 31·5 mg/kg ip in rats. In rabbits cutaneous application of 500 mg/kg was lethal but all those tested survived 100 mg/kg, although both doses produced marked skin changes. In subacute toxicity tests, oral dosage of rats with up to 100 mg/kg five times weekly for 6 wk was without effect, but when the dose was increased to 200 mg/kg apathy, dyspnoea, impaired quality of coat and a tendency to fall appeared. Rats on this high dose rapidly showed changes in the gastric mucosa, inflammation of the intestinal mucosa and swollen mesenteric lymph nodes; in females the adrenals were enlarged. Rats on lower doses showed none of these changes. Patch tests on rabbits were negative after single or repeated applications of 0·5% Bronidox in oily solution. Multiple applications of 0·5% in soft paraffin or oil-in-water or water-in-oil creams to hairless mice and rats produced local necrosis or exfoliation in some animals, while a single application of 2·5% in soft paraffin consistently produced necrosis. In the rabbit eye, 0·05% Bronidox instilled daily had no effect, but a single instillation of 0·5% produced redness, swelling and purulent exudation. No sensitization was induced in guinea-pigs by intradermal injection of 1 or 2·5% Bronidox in oil.

In rats and rabbits the major urinary metabolites of the preservative were 2-bromo-2-nitropropan-1,3-diol and 2-nitroethanol. In man, 10·8–75·3% of a dose of Bronidox applied to the skin in a cream formulation appeared in the urine within 4 hr. In human tolerance tests, a 0·05% cream applied for 21 days had no effect, but patch tests with 0·5% creams or ointments sometimes produced a marked reaction. An in-use test of shampoo and foam-bath formulations indicated that Bronidox is safe for use in the proposed antimicrobial concentration of 0·05%.

3217. The fate of an anti-dandruff agent

Klaassen, C. D. (1976). Absorption, distribution, and excretion of zinc pyridinethione in rabbits. *Toxic. appl. Pharmac.* **35**, 581.

When zinc pyridinethione (ZPT) labelled with ^{35}S was given orally or ip to rats, rabbits or monkeys, the major part was rapidly excreted in the urine, although 15% appeared in the bile and 2–9% still remained in the body tissues after 72 hr (Ziller, *Fd Cosmet. Toxicol.* 1977, **15**, 49). The findings with sodium pyridinethione were comparable and some 1–2% of the sodium salt (again ^{35}S-labelled) was similarly eliminated in the bile after iv administration to rats, but after its application to rat or monkey skin, biliary excretion was insignificant and only about 2% appeared in the urine (Parekh, *ibid* 1970, **8**, 147). Correlating with this apparently low degree of percutaneous absorption are the negative results obtained in several dermal toxicity studies. No evidence of embryotoxicity or teratogenicity followed the repeated dermal application to pregnant rabbits of a shampoo containing ZPT at dose levels of 20 or 50 mg/kg/day (*Cited in F.C.T.* 1976, **14**, 366). Although repeated oral doses of this order or less produced muscle paralysis in rabbits and rats and blindness in dogs, as much as 200 mg ZPT/kg applied to the skin of rabbits for 3 months was without any detectable

adverse effect (*ibid* 1966, **4,** 554). Further comparative evidence on the fate of ZPT when given by different routes is now presented.

Groups of three rabbits were given ^{14}C- or ^{65}Zn-labelled ZPT at a dose level of 1 mg/kg iv or 40 mg/kg by the oral or dermal routes, and radioactivity in the blood, urine and tissues was determined over the following 6–8 hr. After iv administration, blood levels of ^{14}C fell rapidly while those of ^{65}Zn remained relatively constant, and 75% of the ^{14}C but only 0·5% of the ^{65}Zn appeared in the urine over 6 hr. After this time only 4·4% of the ^{14}C but as much as 55% of the ^{65}Zn remained in the major organs, and 0·6% of the ^{65}Zn but less than 0·03% of the ^{14}C remained in the blood. The concentration of ^{65}Zn in the whole blood was much higher than that in the plasma, whereas ^{14}C concentrations in whole blood and plasma were nearly equal. Six hours after oral administration only 1·35% of the ^{14}C and 4·4% of the ^{65}Zn were found in the major organs, and the urine contained 45% of the ^{14}C and 0·03% of the ^{65}Zn. Levels of ^{14}C in the blood decreased after 2 hr while those of ^{65}Zn increased, and after 6 hr about 0·01% of the ^{14}C and 0·0035% of the ^{65}Zn remained in the blood. When ZPT was applied dermally for 4 hr only 0·49% of the ^{14}C and 0·007% of the ^{65}Zn could be detected in the major organs after 8 hr, while the blood contained less than 0·0004% of the ^{14}C and 0·00009% of the ^{65}Zn, and the amount excreted in the urine corresponded to only 0·5% of the ^{14}C and less than 0·002% of the ^{65}Zn.

The findings showed that the organic and inorganic portions of ZPT dissociate prior to distribution in and excretion from the body, the former being excreted far more rapidly than the latter. This may be due to metabolism to elemental zinc and incorporation into the zinc body pool. Only a small percentage of either portion penetrates the skin, and the zinc does so to a lesser extent than the organic portion. There was no excessive accumulation of either ^{65}Zn or ^{14}C in the muscle or eye, and the part of the molecule responsible for the toxic effects of ZPT therefore still remains to be determined.

METHODS FOR ASSESSING TOXICITY

3218. It's no monkey business

Litterst, C. L., Gram, T. E., Mimnaugh, E. G., Leber, P., Emmerling, D. & Freudenthal, R. I. (1976). A comprehensive study of *in vitro* drug metabolism in several laboratory species. *Drug. Metab. Dispos.* **4,** 203.

In recent years the biomedical research community within the United States has made use of approximately two-thirds of the non-human primates imported into the country. It is now apparent that there is an acute shortage of the rhesus monkey (*Macaca mulatta*), which has frequently been used as the primate model for preclinical drug testing. Although the situation may be somewhat alleviated in the future by the provision of new breeding colonies, attempts are being made to tackle the immediate problem by identifying appropriate alternative species.

The present study, the first in a series designed to evaluate animal species for laboratory use, compares the drug-metabolizing capacity *in vitro* of the rhesus monkey (*M. mulatta*), squirrel monkey (*Saimiri sciureus*), Hanford miniature pig, common tree shrew (*Tupaia glis*) and Sprague-Dawley-derived rat. The activities of microsomal and soluble enzymes from homogenized livers were determined by aerobic incubation at 37°C. Enzymes assayed were NADPH-cytochrome c reductase, ethylmorphine *N*-demethylase, aminopyrine *N*-demethylase, aniline hydroxylase, biphenyl hydroxylase, benzopyrene hydroxylase, glutathione *S*-aryltransferase, UDP-glucuronyltransferase and *N*-acetyltransferase.

No single species resembled the rhesus in all the quantitative aspects of its *in vitro* drug metabolism, although all species showed qualitative similarity to the monkey. Using an arbitrary scale of favourable response and recording the number of times that a species responded similarly to the rhesus, an order of favourable comparisons was obtained, pig > rat > squirrel monkey > tree shrew. Although the miniature pig appears to possess a drug-metabolizing system that resembles that of the rhesus, difficulties in the housing and handling of this animal could reduce its value in a general drug-development programme. Results for the squirrel monkey, which can be assumed to be the test species most closely related phylogenetically to the rhesus, surprisingly showed the fewest absolute similarities to the rhesus. This observation suggests that a primate replacement for the rhesus may not necessarily be requisite or even particularly desirable. It should be pointed out, however, that these two species of monkey represent two distinct phylogenetic groups, for which differences in drug metabolism have previously been demonstrated. With the possibility of establishing breeding colonies, the tree shrew shows potential for use in drug testing. In this species, a sex difference was observed in three pathways of drug metabolism in both microsomal and soluble enzymes. In contrast to the situation in the rat, however, the female appears to be a more active metabolizer of drugs than the male.

The authors concluded that any of the four species examined could substitute adequately for the rhesus monkey with respect to drug metabolism *in vitro*. All exhibited the ability to metabolize drug substrates and differed only with regard to the rates at which the drugs were metabolized. Subsequent publications in this series will examine the *in vivo* toxicity, *in vivo* metabolism and pharmacokinetics of antineoplastic drugs in these five species.

3219. Mussels as mutagen monitors

Parry, J. M., Tweats, D. J. & Al-Mossawi, M. A. J. (1976). Monitoring the marine environment for mutagens. *Nature, Lond.* **264,** 538.

Monitoring the marine environment by means of mutagenicity tests could give some indication of pollution levels, and the above workers have developed a biological assay system to detect such pollutants. The mussel *Mytilus edulis* is widespread in coastal waters and because it feeds by filtering sea-water and has an intracellular mode of digestion. it tends to accumulate pollutants in its tissues. which provide testable material.

Alcoholic extracts were prepared from the tissues of mussels collected from Plymouth (an area of heavy visible pollution), Mumbles, Caswell Bay and Anglesey (a 'clean-water area'). Samples from liver and from non-liver tissues were tested separately in the case of the Mumbles mussels. Test samples were screened using auxotrophic yeast cultures to detect induction of prototrophic colonies produced by mitotic gene conversion (a non-specific response to mutagens and carcinogens). Exposure of yeast cultures auxotrophic for histidine and tryptophan to mussel extracts at concentrations up to 4% produced significant increases in both histidine and tryptophan prototrophs, except with mussel extracts from the clean waters of Anglesey. Most of the genetic activity was shown to be in the non-liver extracts.

Extracts were also exposed to cultures of both *Salmonella typhimurium* (in a slightly modified Ames test) and *Escherichia coli* carrying genetic markers to detect forward and reverse mutation by both base-substitution and frameshift DNA changes. *E. coli* was used in plate incorporation tests and in modified fluctuation tests. In the latter, Mumbles extracts increased the mutation frequency in bacterial strains responsive to both frame-shift and base-change mutagens or to base-change mutagens only, but not in a strain carrying a frame-shift mutation. The non-liver extracts of the mussels again carried most genetic activity. Similar results were obtained with *E coli* strains in plate incorporation tests, but with *S. typhimurium* the mussel extracts increased mutation frequency in a strain producing prototrophs by frameshift mutation but not in one producing prototrophs by base substitution. This was considered to reflect the specificity of the strains used (and underlined the need to use a large number of strains in such tests). Control experiments showed that the mussel extracts did not contain nutrient supplements or radioactive materials.

The authors conclude that these results provide convincing evidence for the concentration of mutagens in the body tissues of mussels, reflecting to some extent the degree of pollution in the local environment. and they suggest that such agents may represent a possible source of carcinogens, although no evidence was obtained regarding the identity of the chemicals.

PATHOLOGY

3220. The shape of the implant problem

Matlaga. B. F., Yasenchak. L. P. & Salthouse, T. N. (1976). Tissue response to implanted polymers: the significance of sample shape. *J. biomed. Mater. Res.* **10**, 391.

The development of plastics materials has allowed a steady increase in the use of artificial implants in human surgery for anatomical, functional or cosmetic reasons. Implant-related basic research has centred mainly on problems of biocompatibility and toxicity (*Cited in F.C.T.* 1967. **5**, 569; *ibid* 1972. **10**, 567). Current ideas on the characteristics and mechanisms involved in foreign-body tumorigenesis are to be found in a comprehensive review by Brand *et al.* (*Crit. Rev. Toxicol.* 1976. **4**, 353). The study cited above examines one aspect of the problem. namely the effects of different shapes of polymeric samples on the tissue response.

Medical-grade polymers of polyvinyl chloride, polyethylene, polypropylene. teflon and silicone rubber were extruded through stainless-steel dies to yield rods with triangular. circular and pentagonal cross-sections. Sterile 15-mm lengths were cut and implanted into the gluteal muscles of female Long Evans rats. All samples had almost identical cross-sectional perimeters so that the cells surrounding the implants were exposed to the same amount of polymer surface. A quantitative method based on the activity of lysosomal acid phosphatase in the macrophage population around the implant was used to measure the cellular response to the implants following 14 days of exposure.

One-way analysis of variance computations indicated statistical differences among sample shapes at the 5% level. The triangular-shaped rods of all types of polymer were associated with the highest activity of lysosomal acid phosphatase in cells surrounding the implant. The pentagon-shaped rods induced less activity, whilst the circular rods induced the lowest activity. Foreign-body reaction at 14 days consisted mainly of macrophage cells with an outer ring of fibroblasts. The greatest proliferation of cells was observed around the triangular-shaped rods.

Although the effects observed may have been the result of a variety of factors, the most probable cause was considered to be implant movement within the muscle. A triangular shape, having the most acute angles, initiated the greatest tissue response.

Rat muscle is not commonly used for implantation studies but was used in the present work because it was large enough to accommodate the samples and induces less variation in response than does rabbit muscle. The authors recognize a need for standardization of sample shapes in order to provide valid comparative results in implantation studies.

CANCER RESEARCH

3221. Dust in respiratory carcinogenesis

Sellakumar, A., Stenbäck, F. & Rowland, J. (1976). Effects of different dusts on respiratory carcinogenesis in hamsters induced by benzo(a)pyrene and diethylnitrosamine. *Eur. J. Cancer* **12**, 313.

Initiation and promotion were proposed as two distinct stages in tumour formation by Berenblum & Shubik (*Br. J. Cancer* 1974, **1**, 383). Initiation is very rapid and is irreversible, producing dormant tumour cells which depend for their further development upon a relatively slow and partly reversible process of promotion. Intratracheal instillation of benzo[*a*]-pyrene (BP) absorbed on ferric oxide (Fe_2O_3) particles produced a number of tumours in hamsters (Saffiotti, *Prog. exp. Tumor Res.* 1969, **11**, 302). Fe_2O_3 has also been found to enhance the tumour-inducing effect of diethylnitrosamine (DEN) (*Cited in F.C.T.* 1976, **14**, 652). In the first experiment described below, an attempt was made to determine whether Fe_2O_3 acted as a promoting agent with DEN in two-stage carcinogenesis or as an initiating agent. In the second experiment, the role of Fe_2O_3 when given with BP was investigated. The promoting effects of carbon and aluminium oxide (Al_2O_3) were also examined.

In the first experiment, random-bred 8- or 9-wk-old male Syrian golden hamsters in groups of 35–48 were treated with 1 mg DEN injected sc in 0·25 ml saline and with Fe_2O_3 given intratracheally in 0·2 ml saline. In group 1, given weekly injections of DEN for 6 wk followed after a 6-wk rest by weekly doses of 3 mg Fe_2O_3 for 15 wk, 63% of the animals showed respiratory tumours, mainly tracheal and laryngeal. For group 2 the treatment was reversed, Fe_2O_3 being given first, and as a result, only 31% of the animals bore tumours, the first tracheal papillomas appearing much later than those in group 1. In group 3, a lower dose of Fe_2O_3 (2 mg) and longer treatment (15 doses given in alternate weeks), followed after a 6-wk break by 6 weekly DEN injections, again produced tumours in 31%, the first tumours appearing even later than in group 2. Single applications of DEN and Fe_2O_3, 1 wk apart (group 4), induced only a single tracheal tumour in one of 35 animals. Simultaneous Fe_2O_3 and DEN administration (group 5) induced laryngeal and tracheal papillomas in 27% of the animals, the first papilloma appearing relatively early (after 27 wk). Six weekly applications of DEN alone produced laryngeal and tracheal papillomas in 58% of group 6, the first papilloma being detected at wk 20. There were no respiratory-tract tumors in untreated animals. Tumours were histologically similar in all groups; laryngeal and tracheal tumours had a papillary configuration while lung tumours were adenosquamous. There was a much higher proportion of adenosquamous tumours in the animals given DEN followed by Fe_2O_3 (group 1) than in the other groups, in agreement with previous studies (*ibid* 1976, **14**, 652), although the overall percentage of tumour-bearing animals did not increase significantly. Each of the group 1 animals with adenosquamous tumours also had tracheal papillomas. This suggests that the adenosquamous tumours may have been auto-transplanted tumours. The fact that simultaneous application of Fe_2O_3 and DEN induced a slightly lower number of tumours of the upper respiratory tract than appeared when Fe_2O_3 was administered after DEN suggests a promoting effect of Fe_2O_3, but whether this was an initiation–promotion process is not yet clear.

In the second experiment, all treatments were given intratracheally in 0·2 ml saline. In group 1, which received 3 mg BP weekly for 10 wk, 15% of animals developed benign tumours of the upper respiratory tract. There were no respiratory tumours in group 2 given Fe_2O_3 alone. Group 3, receiving BP and Fe_2O_3 simultaneously for 10 wk, produced the highest proportion of tumour-bearing animals (71%). Instillation of BP followed by Fe_2O_3 (group 4) and Fe_2O_3 followed by BP (group 5) each induced a similar tumour incidence (13% of animals in each case). Replacing the Fe_2O_3 of group 4 with Al_2O_3 or with carbon resulted in about half the number of tumours. Again, there were no respiratory-tract tumours in untreated controls. Groups treated with BP showed higher incidences of forestomach papillomas than did untreated controls. From a comparison of the incidence of tumours in group 4 with that in group 1, it is concluded that Fe_2O_3 did not act as a promoter, nor did Al_2O_3 or carbon dusts. The increased incidence of tumours in group 3, following simultaneous administration of BP and Fe_2O_3, supports the view of Fe_2O_3 as a co-carcinogen, and may have resulted from an increase in retention of BP when it was applied simultaneously with Fe_2O_3.

3222. A picture of dimethylnitramine carcinogenicity

Goodall, C. M. & Kennedy, T. H. (1976). Carcinogenicity of dimethylnitramine in NZR rats and NZO mice. *Cancer Lett.* **1**, 295.

Dimethylnitrosamine is a recognized carcinogen. The replacement of its nitroso group by a nitro group yields dimethylnitramine, which has been tested for carcinogenicity in an attempt to shed some light on the mechanism of action of the environmentally important *N*-nitroso compounds.

Mice were injected sc at birth with 100 μg dimethylnitramine and subsequently were given ip injections of 200 μg of the amine at 12-day intervals, so that each received a total dose of 70 mg over 204 days. Thereafter the animals received drinking-water containing 100 mg dimethylnitramine/litre. The mice showed a clear carcinogenic effect, with an 81% incidence of liver carcinomas and a 48% incidence of adenocarcinomas of the renal tubular epithelium. In addition, the incidence of lymphomas, lung tumours and duodenal adenomas was significantly increased in dimethylnitramine-treated mice. In another experiment, rats were given drinking-water containing 30 mg dimethylnitramine/litre for 1 yr from 35 days of age and were subsequently given plain water until death.

The main targets for dimethylnitramine carcinogenesis appear, therefore, to be the liver epithelial cells and, with larger doses, the renal tubular epithe-

lium. The morphology of the liver tumours induced by dimethylnitramine differed from that of tumours induced by dimethylnitrosamine, suggesting that the two compounds probably have different mechanisms of action and may not be interconvertible in the body.

3223. A stomachful of nitrosocarbaryl

Lijinsky, W. & Taylor, H. W. (1976). Carcinogenesis in Sprague-Dawley rats of *N*-nitroso-*N*-alkylcarbamate esters. *Cancer Lett.* **1**, 275.

Eisenbrand, G., Schmähl, D. & Preussmann, R. (1976). Carcinogenicity in rats of high oral doses of *N*-nitrosocarbaryl, a nitrosated pesticide. *Cancer Lett.* **1**, 281.

The nitrosation of carbaryl and other *N*-methylcarbamate pesticides in the environment is a distinct possibility (Elespuru & Lijinsky, *Fd Cosmet. Toxicol.* 1973, **11**, 807). *N*-Nitrosocarbaryl (NC) has been shown to produce local sarcomas after sc injection into rats (Eisenbrand *et al. ibid* 1975, **13**, 365) and work now reported demonstrates the carcinogenicity of this compound when administered by the more relevant oral route.

The first paper cited above describes experiments in which groups of 12 female rats were given NC, nitrosomethylurethane or nitrosoethylurethane by gavage in the form of 0·2 ml of a 0·11 M solution in olive oil once-weekly for 10 wk, to provide total doses of 50, 29 and 32 mg, respectively. Both NC and the methylurethane derivative produced tumours, mostly squamous-cell carcinomas of the forestomach, in 75–80% of the animals, earlier death being associated with the second compound. All the animals given the ethylurethane derivative developed squamous cancers of the stomach. Thirteen male rats given a total of 300 mg NC during a 20-wk period developed no more stomach tumours than females on the lower dose, but they died earlier.

The second paper describes a study using 31 male rats given 130 mg NC/kg twice-weekly for life. In 29% of the animals, death with a squamous-cell carcinoma of the forestomach occurred after an average of 167 days; the first showing this type of tumour died only 63 days from the start of treatment. A further 19% of the animals had hyperkeratoses and 6% had papillomas of the forestomach. No tumours were observed in other organs. The two lymphosarcomas and one leukaemia that developed in the 29 untreated control rats appeared between days 300 and 650. The treated rats that were found to have hyperkeratoses or papillomas died very early, and these lesions are regarded as an early indication of the carcinogenic effect of NC. Survival curves show that at the time when the incidence of tumours was increasing most rapidly (after 200 days) most of the treated rats were already dead, indicating that the cumulative toxic effects of such a high dose of NC, rather than tumour development, killed a high proportion of these animals. Both of the studies cited, however, confirm NC as a potent locally-acting carcinogen when taken by mouth.

[The studies considered in this and the preceding abstract are all concerned with the important question of the carcinogenicity of nitroso compounds, which are acquiring increasing recognition as environmental contaminants. Regrettably two of these studies appear to have relied on 'historical' control data for demonstrating an increase in tumour incidence as a result of treatment. While comparisons of this sort may be a valid and useful aid in the evaluation of apparent but not statistically significant differences between test animals and concurrent controls, they cannot replace concurrent control data for two reasons. The first is that tumour incidence in animals varies from generation to generation and this may give a false base-line for deductions. This is particularly the case with mice but is also relevant to other species. Secondly, it is unlikely that histological diagnosis of both test and historical control tissues will have been carried out by the same pathologist and there is therefore a strong possibility of significant differences in the classification of the tumours observed.]

Fd Cosmet. Toxicol. Vol. 15, p. 501. Pergamon Press 1977. Printed in Great Britain

FORTHCOMING PAPERS

It is hoped to publish the following research papers in the next issue of *Food and Cosmetics Toxicology*:

Long-term toxicity study of Black PN in mice. By J. J.-P. Drake, K. R. Butterworth, I. F. Gaunt and P. Grasso.

Short-term study in the rat on two caramels produced by variations of the 'ammonia process'. By I. F. Gaunt, A. G. Lloyd, P. Grasso, S. D. Gangolli and K. R. Butterworth.

Long-term toxicity study in the rat on a caramel produced by the 'half open–half closed pan' ammonia process. By J. G. Evans, K. R. Butterworth, I. F. Gaunt and P. Grasso.

Long-term effects of calcium carrageenan in rats. I. Effects on reproduction. By T. F. X. Collins, T. N. Black and J. H. Prew.

Long-term effects of calcium carrageenan in rats. II. Effects on foetal development. By T. F. X. Collins, T. N. Black and J. H. Prew.

Some observations on the determination of monomer residues in foods. By J. C. Chudy and N. T. Crosby.

Citrinin mycotoxicosis in the guinea-pig. By H. L. Thacker, W. W. Carlton and G. A. Sansing.

Ochratoxin A mycotoxicosis in the guinea-pig. By H. L. Thacker and W. W. Carlton.

Acute oral toxicity of ethylidene gyromitrin in rabbits, rats and chickens. By S. M. Mäkinen, M. Kreula and M. Kauppi.

Toxicity of a moniliformin-producing strain of *Fusarium moniliforme* var. *subglutinans* isolated from maize. By N. P. J. Kriek, W. F. O. Marasas, P. S. Steyn., S. J. van Rensburg and M. Steyn.

Leaching of radionuclides from neutron-activated talc in serum and in dilute hydrochloric acid. By C. L. Wilkerson, A. P. Wehner and L. A. Rancitelli.

The absorption of *p*-toluenediamine by the skin of rats and dogs. By R. Hruby.

Non-carcinogenicity of hair dyes: Lifetime percutaneous applications in mice and rabbits. By F. G. Stenbäck, J. C. Rowland and L. A. Russell.

Assessment of the acute toxicity and potential irritancy of hair dye constituents. By G. K. Lloyd, M. P. Liggett., S. R. Kynoch and R. E. Davies. (Short paper)

Fd Cosmet. Toxicol. Vol. 15, p. 502. Pergamon Press 1977. Printed in Great Britain.

CORRIGENDA

Volume 15 (1977)

p. 246, line 49 (right-hand column): *For* suppl. 246) *read* suppl. 246, p. 1).
p. 260, line 6 (right-hand column): *For* Douglas, D. E., *read* Douglas D. R.
p. 337, line 27 (right-hand column): *For* 12, 16 and 20 of gestation *read* 12, 15 and 20 of gestation.
p. 367, line 30 (left-hand column): *For* Inouse *read* Inoue.

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Aims and Scope

The Journal provides a wide-ranging service of general articles and abstracts on food and cosmetics toxicology. The Journal is primarily intended for the use of food scientists and technologists, manufacturers and administrators who neither read nor have access to the medical, pharmacological or toxicological literature. In addition, the journal will constitute a medium for the publication of reviews and original articles relating to the fields of interest covered by the British Industrial Biological Research Association.

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e.g. Hickman, J. R., McLean, D. L. A. & Ley, F. J. (1964). Rat feeding studies on wheat treated with gamma-radiation. **1.** 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.*:

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