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

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Research Section

DISTRIBUTION AND EXCRETION OF TWO PHTHALATE ESTERS IN RATS, DOGS AND MINIATURE PIGS

G. J. IKEDA, P. P. SAPIENZA, J. L. COUVILLION, T. M. FARBER, C. P. SMITH, P. B. INSKEEP, E. M. MARKS, F. E. CERRA and E. J. VAN LOON

Division of Toxicology, Food and Drug Administration, Washington, DC 20204, USA

(*Received 10 April 1978*)

Abstract—Diisooctyl phthalate (DIOP) or butylglycolylbutyl phthalate (BGBP) was administered in the diet to male Sprague-Dawley rats, beagle dogs and miniature pigs in a dose of 50 mg/kg for 21-28 days before oral administration of a single dose of the same compound labelled with ^{14}C in the carbonyl group. The animals were killed and tissues, faeces and urine were analysed for ^{14}C at various times after the dose of [^{14}C]phthalate was administered. In the rat, approximately half of the ^{14}C activity from labelled DIOP was excreted in the urine and half in the faeces, while radioactivity from this phthalate appeared predominantly in the faeces in dogs (69-80%) and in the urine in pigs (65-86%). The BGBP label was excreted mainly in the urine in rats (77-83%), dogs (72-75%) and pigs (68-89%). Extraction of the tissues for metabolites indicated that DIOP was less readily metabolized than BGBP by all three species. The nature of the substituent on the phthalate molecule appears to influence the excretion of the phthalate-ester metabolites.

INTRODUCTION

Phthalic acid esters are used extensively as plasticizers. Since plastics are widely used in food packaging, phthalate esters become food contaminants and, as such, are ingested by man. Studies on the distribution, excretion and metabolism of phthalate esters have been performed in rats (Albro & Moore, 1974; Albro, Thomas & Fishbein, 1973; Daniel & Bratt, 1974; Rowland, 1974; Rowland, Cottrell & Phillips, 1977; Schulz & Rubin, 1973; Williams & Blanchfield, 1974), and distribution and excretion studies have been reported in trout (Melancon & Lech, 1976). Rats were found to excrete metabolites of di-(2-ethylhexyl) phthalate rapidly, mainly in the urine, and fish have been reported to excrete much of the dose in the bile (Melancon & Lech, 1976).

In order to explore further the fate of phthalate esters in mammalian systems, we have examined the distribution and excretion of two phthalate esters, technical diisooctyl phthalate (DIOP) and butylglycolylbutyl phthalate (BGBP), in Sprague-Dawley rats, beagle dogs and miniature pigs.

One objective of this study was to determine whether a given phthalate ester would be handled similarly in three different mammalian systems, i.e. a rodent and two large species. This paper will show that, while the two phthalate esters are generally not persistent compounds, they do differ to some extent in their distribution and excretion within a species. Differences are also apparent when the same compound is administered to three different species.

EXPERIMENTAL

Chemicals. Technical DIOP was a product of EXXON Chemical Co., Linden, NJ. The typical isomer distribution of the alcohol used in the synthesis of the ester was 20% 3,4-dimethyl-1-hexanol, 30% 3,5-dimethyl-1-hexanol, 30% 4,5-dimethyl-1-hexanol, 15% 3- and 5-methyl-1-heptanols and 5% unidentified alcohols. The labelled DIOP was the diester of 3,5-dimethyl-1-hexanol, labelled with ^{14}C (12.1 mCi/mmol) in the carbonyl group and was synthesized by Mallinckrodt Labeled Compounds, St. Louis, MO. Radiochemical purity was ≥98%. The BGBP used was Santicizer B-16, a product of Monsanto Co., St. Louis, MO. According to the manufacturer, a typical composition of commercial BGBP was 92% BGBP, 5% dibutyl phthalate, 2% dibutylglycolyl phthalate and 0·2% 1-butanol. [*Carbonyl*-^{14}C]BGBP (11·2 mCi/mmol) was synthesized by Mallinckrodt Labeled Compounds, and was claimed to be 98% radiochemically pure.

Animals and diets. Throughout the experiments, adult male Sprague-Dawley rats weighing 200-300 g, were maintained on Purina Ground Laboratory Chow, pure-bred male beagles, approximately 1 yr old and weighing 7-10 kg, were maintained on Purina Laboratory Canine Diet, and male miniature swine of the Hormel strain, 4-12 months old and weighing 10-25 kg, were maintained on SR No. 3 Diet (obtained from the US Department of Agriculture, Agricultural Research Center, Beltsville, MD). The SR No. 3 diet contained 16% protein, 75% carbohydrate and 2·9% fat. Tap-water was available *ad lib.*

Dosing. All animals were pretreated orally with the appropriate phthalate ester, a dose of 50 mg/kg being added daily to the diet for 21-28 days prior to administration of the radioactive dose. The animals were fasted overnight before administration of the radioactive compound. A corn-oil solution of the labelled phthalate, approximately 5 μCi/kg body weight, along with an appropriate amount of unlabelled ester to bring the dosage to the 50-mg/kg level was administered by gastric tubing or in a gelatin capsule. The

latter technique, which was used in about one quarter of the experiments with dogs and pigs, minimized dosing problems.

Metabolic experiment. After receiving the radioactive dose, the animals were placed in individual metabolism cages designed to permit separate collection of urine and faeces. For experiments longer than 24 hr, the animals were fed diet containing the appropriate ester at 50 mg/kg until they were killed. Urine and faeces were collected at least once during each 24-hr period and at the end of the experiment.

Sample collection and analysis. Pigs and dogs were bled at various times after the administration of the ^{14}C dose to determine the blood elimination half-lives. At the end of experimental period, three to six rats were killed by cervical dislocation and exsanguination, while the three to five dogs and three to five pigs were killed by electrocution followed by immediate exsanguination. The various organs and tissues were removed, washed, blotted, weighed and prepared for analysis. Bile and blood samples were collected at autopsy.

Urine samples were assayed for ^{14}C by counting in a liquid scintillation cocktail. Faeces and tissue samples were homogenized when necessary, and weighed samples were oxidized to $^{14}CO_2$ in a biological sample oxidizer (Packard Model 305). Bile samples were also oxidized in the biological sample oxidizer. The dissolved $^{14}CO_2$ samples were assayed for radioactivity by liquid scintillation counting (Packard Model 3390).

In order to determine the proportion of the parent compound metabolized, the urine, faeces and tissue samples were also homogenized in chloroform-methanol (2:1, v/v) and extracted with 0·1–0·2 mM-aqueous NaOH (pH 10). Samples of each of these layers were assayed for radioactivity by liquid scintillation counting.

Aliquots of tissue extracts and urine samples were pipetted into low-potassium glass vials. Chloroform-methanol extracts were dried after pipetting to remove the solvent and eliminate chemical quenching. Liquid scintillation cocktail, either Instagel® (Packard Instrument Co., Chicago, IL) or a mixture of Triton X-100 and toluene (33:67, v/v) containing 5·5 g 2,5-diphenyloxazole (PPO) and 125 mg 1,4-bis-2-(4-methyl-5-phenyloxazolyl)benzene (dimethylPOPOP)/litre, was added to each vial; the samples were mixed well and counted in the liquid scintillation counter. Either an internal or an external standard was used to correct for quenching.

RESULTS

The excretion of ^{14}C and the distribution of residual ^{14}C in the tissues of rats, dogs and pigs at various times after the adminstration of a single oral dose of [^{14}C]DIOP are presented in Tables 1, 2 and 3. At 24 hr, about 85% of the dose was found in the urine and faeces in rats, while only about 50% of the dose was found in the urine and faeces of dogs and pigs. Radioactivity persisted in the gastro-intestinal tract over a period of several days in all species, and was detected in bile samples of both dogs and pigs during this same period. The level of ^{14}C in dog bile was approximately 25 times higher than that in pig bile.

The pig excreted most of the ^{14}C from DIOP in the urine (65–86%), while the dog excreted most of the ^{14}C in the faeces (69–80%). Excretion in the rat was intermediate, approximately half of the dose being in the urine and half in the faeces.

Some of the administered radioactivity was distributed to body fat in all three species. However, distribution of radioactivity to lipid-rich tissues such as the brain and the lung was minimal. Excretion of [^{14}C]DIOP was virtually complete in 4 days in rats. Slightly more than 4 days were required for complete excretion of the ester in dogs, and pigs required nearly 21 days in order to excrete the ester completely.

The blood elimination half-lives of [^{14}C]DIOP in the dog and pig were 1·2 and 5·4 hr, respectively. On this basis the elimination rate of ^{14}C in dogs was four to five times that in pigs, and the excretion data also indicated more rapid elimination in the dog than in the pig.

When dosed with 50 mg BGBP/kg, rats excreted a large fraction of the administered ^{14}C in the urine

Table 1. *Distribution and excretion of radioactivity following oral administration of diisooctyl [^{14}C]phthalate (50 mg/kg in corn-oil solution) to male Sprague–Dawley rats*

		Mean values* for animals killed at			
		4 hr	8 hr	24 hr	4 days
Sample†	No. of rats...	5	6	4	5
Liver		3·89 ± 0·21	2·00 ± 0·17	0·24 ± 0·05	+‡
Fat		1·16 ± 0·39	0·56 ± 0·19	0·11 ± 0·01	+
Lung		0·13 ± 0·01	0·03 ± 0·00	+	+
Kidney		0·94 ± 0·06	1·17 ± 0·49	0·07 ± 0·02	+
Gastro-intestinal tract		79·50 ± 5·02	65·29 ± 5·83	9·56 ± 3·19	+
Urine		ND	13·78 ± 2.22	40·64 ± 4.11	57·06 ± 6·67
Faeces		ND	6·34 ± 2·22	44·98 ± 6·48	38·17 ± 8·05
Recovery (% of dose)...		87·56 ± 5·70	88·50 ± 4·35	97·25 ± 5·39	95·24 ± 3·23

ND = Not detected

*Values are mean percentages of the dose ± SEM/whole organ or total urine or faeces, with the exception of those for fat, which are mean percentages of the dose ± SEM/g tissue.

†Brain, muscle and bile samples showed no radioactivity.

‡Present, but in amounts too small to be estimated (<0·01% of dose/g sample).

Table 2. *Distribution and excretion of radioactivity following oral administration of diisooctyl [^{14}C]phthalate (50 mg/kg in corn-oil solution) to male beagle dogs*

Sample	No. of dogs...	Mean values* for animals killed at 4 hr	24 hr	4 days	21 days
		5	4	4	4
Liver		3·62 ± 0·56	0·84 ± 0·27	0·05 ± 0·02	0·03 ± 0·00
Fat		0·44 ± 0·16	0·22 ± 0·03	0·02 ± 0·02	0·12 ± 0·02
Brain		0·02 ± 0·00	+†	+	+
Lung		0·14 ± 0·03	+	+	+
Muscle		3·58 ± 1·00	0·27 ± 0·13	0·31 ± 0·23	0·07 ± 0·01
Kidney		0·34 ± 0·10	0·04 ± 0·00	+	+
Bile		12·92 ± 3·39	6·28 ± 2·64	0·29 ± 0·15	+
Gastro-intestinal tract		60·98 ± 1·28	35·41 ± 10·46	0·53 ± 0·22	0·06 ± 0·01
Urine		9·24 ± 0·10	9·44 ± 2·30	23·28 ± 1·84	27·77 ± 7·40
Faeces		ND	41·08 ± 14·68	80·02 ± 2·44	68·55 ± 5·63
	Recovery (% of dose)...	94·25 ± 3·99	94·50 ± 5·12	104·75 ± 2·17	96·75 ± 3·17

ND = Not detected

*Values are mean percentages of the dose ± SEM/whole organ or total urine or faeces. Fat was estimated to be 1% of body weight.

†Present, but in amounts too small to be estimated (<0·01% of dose/g sample).

and approximately 10% in the faeces during day 1 (Table 4). The dose was almost completely excreted by day 4. When administered to dogs, three quarters of the radioactivity appeared in the urine in 24 hr (Table 5). The amounts of the dose excreted in the urine and faeces did not vary significantly when the experiment was prolonged to 21 days. The amount of ^{14}C secreted in the bile declined rapidly. A small amount of the dose was distributed to fat, and the amounts distributed to lipid-rich tissues such as the lung and brain were not extensive. The excretion pattern of BGBP in pigs (Table 6) did not appear to differ from that in dogs; approximately three quarters of the administered radioactivity again appeared in the urine and the dose was virtually eliminated in 4 days.

The blood elimination half-lives of [^{14}C]BGBP in the dog and pig were 1·2 and 2·2 hr, respectively. These short half-lives agree with the results of the excretion studies, which indicated rapid elimination in the urine in both species.

Aqueous-organic solvent partitions of tissues and excreta were performed in order to determine the amount of metabolized compound that could be found in the samples at a given point in time. These results appear in Table 7. At any given time, the highest rate of metabolism was displayed by rat tissue. DIOP appeared to be less readily metabolized than BGBP in all three species.

DISCUSSION

The radioactivity from [^{14}C]DIOP persisted over a period of several days in the gastro-intestinal tract and bile of both dogs and pigs. This appears to indicate enterohepatic recycling of DIOP and/or its meta-

Table 3. *Distribution and excretion of radioactivity following oral administration of diisooctyl [^{14}C]phthalate (50 mg/kg in corn-oil solution) to male miniature pigs*

Sample	No. of pigs...	Mean values* for animals killed at 4 hr	24 hr	4 days	21 days
		4	3	5	5
Liver		1·43 ± 0·16	0·41 ± 0·05	0·02 ± 0·00	+†
Fat		1·64 ± 0·46	1·45 ± 0·55	0·46 ± 0·12	0·30 ± 0·08
Brain		+	+	+	+
Lung		0·09 ± 0·02	0·03 ± 0·02	+	+
Muscle		1·88 ± 0·66	0·63 ± 0·21	0·06 ± 0·03	0·21 ± 0·10
Kidney		0·43 ± 0·05	0·09 ± 0·01	+	+
Bile		0·73 ± 0·21	0·25 ± 0·13	0·01 ± 0·00	+
Gastro-intestinal tract		74·73 ± 3·73	47·99 ± 6·87	1·58 ± 0·55	0·42 ± 0·17
Urine		14·84 ± 0·75	49·13 ± 3·04	64·59 ± 7·03	86·32 ± 5·36
Faeces		ND	0·13 ± 0·12	32·14 ± 6·24	13·28 ± 3·66
	Recovery (% of dose)...	97·68 ± 4·12	101·00 ± 3·06	99·08 ± 3·02	105·37 ± 1·82

ND = Not detected

*Values are mean percentages of the dose ± SEM/whole organ or total urine or faeces. Fat was estimated to be 22·1% of body weight.

†Present, but in amounts too small to be estimated (<0·01% of dose/g sample).

Table 4. *Distribution and excretion of radioactivity following oral administration of butylglycolylbutyl [^{14}C]phthalate (50 mg/kg in corn-oil solution) to male Sprague–Dawley rats*

	Mean values* for animals killed at			
	4 hr	8 hr	24 hr	4 days
Sample† No. of rats...	4	3	4	6
Liver	1·79 ± 0·19	0·71 ± 0·13	0·15 ± 0·04	+‡
Fat	ND	0·83 ± 0·52	0·02 ± 0·00	+
Lung	ND	0·08 ± 0·03	+	+
Muscle	ND	ND	ND	+
Kidney	ND	0·34 ± 0·05	0·03 ± 0·00	+
Gastro-intestinal tract	65·08 ± 5·03	21·23 ± 6·55	5·50 ± 2·18	+
Urine	21·07 ± 5·86	63·33 ± 1·56	83·38 ± 4·97	76·85 ± 3·23
Faeces	1·76 ± 1·37	1·89 ± 1·67	9·27 ± 1·84	13·25 ± 1·97
Recovery (% of dose)...	85·00 ± 5·82	88·60 ± 4·39	98·25 ± 2·59	89·95 ± 1·88

ND = Not detected

*Values are mean percentages of the dose ± SEM/whole organ or total urine or faeces, with the exception of those for fat, which are mean percentages of the dose ± SEM/g tissue.

†Brain, muscle and bile samples showed no radioactivity.

‡Present, but in amounts too small to be estimated (<0·01% of dose/g sample).

bolites in these species. In contrast, the data in Tables 5 and 6 suggest that extensive enterohepatic recycling does not appear to take place with BGBP. These observations are in line with the fact that, in all three species, BGBP appears to be eliminated more rapidly than DIOP.

The first step in the metabolism of phthalate esters in rats is believed to involve the hydrolysis of one of the alkyl chains to form a monoester (Rowland *et al.* 1977). Because of its free carboxyl group, this monoester can be extracted into aqueous alkali while the neutral diester remains in the organic phase. Aqueous–organic solvent extraction was thus used as a measure of the extent of metabolism of the phthalate esters.

The results in Table 7 indicate that virtually all (96%) of the substance found in rat tissue and excreta 4 days after administration of DIOP was in the form of metabolites, while in the dog and pig, only 63 and 71%, respectively, had been metabolized in 4 days. In all three species, most of the dose of BGBP was converted to metabolite(s) within a 24-hr period, and all of the administered compound was metabolized in 4 days. Apparently the structural differences in the ester side chains of DIOP and BGBP account for the difference in the rates of metabolism and excretion of these phthalate esters. DIOP appears to be distributed to a greater extent in body fat in all three species and retained therein for a longer period of time than BGBP.

Metabolism facilitates excretion of a xenobiotic substance. Thus it is not surprising that a large proportion (approximately 75%) of the radioactivity from an oral dose of BGBP is excreted via the urine in all three species within 24 hr, whereas substantial amounts of radioactivity (as the unmetabolized compound) are present in the tissues of dogs and pigs 1 and 4 days after administration of DIOP. Here

Table 5. *Distribution and excretion of radioactivity following oral administration of butylglycolylbutyl [^{14}C]phthalate (50 mg/kg in corn-oil solution) to male beagle dogs*

	Mean values* for animals killed at			
	4 hr	24 hr	4 days	21 days
Sample No. of dogs...	4	4	3	3
Liver	2·91 ± 0·13	0·24 ± 0·03	+†	0·03 ± 0·02
Fat	0·27 ± 0·07	0·01 ± 0·00	+	+
Brain	0·03 ± 0·00	+	+	+
Lung	0·16 ± 0·02	+	+	+
Muscle	3·52 ± 0·32	0·56 ± 0·16	+	0·04 ± 0·03
Kidney	0·75 ± 0·09	0·06 ± 0·00	+	+
Bile	6·28 ± 1·22	1·13 ± 0·40	0·04 ± 0·01	+
Gastro-intestinal tract	22·46 ± 2·86	4·93 ± 1·46	0·06 ± 0·06	+
Urine	36·60 ± 8·62	74·24 ± 6·82	74·64 ± 0·51	72·25 ± 3·09
Faeces	ND	17·96 ± 2·97	19·25 ± 4·86	21·12 ± 4·92
Recovery (% of dose)...	75·78 ± 4·27	99·27 ± 6·44	93·98 ± 5·05	93·65 ± 5·48

ND = Not detected

*Values are mean percentages of the dose ± SEM/whole organ or total urine or faeces. Fat was estimated to be 1% of body weight.

†Present, but in amounts too small to be estimated (<0·01% of dose/g sample).

Table 6. *Distribution and excretion of radioactivity following oral administration of butylglycolylbutyl [^{14}C]phthalate (50 mg/kg in corn-oil solution) to male miniature pigs*

Sample		Mean values* for animals killed at			
		4 hr	24 hr	4 days	21 days
	No. of pigs...	3	4	3	3
Liver		1·13 ± 0·03	0·33 ± 0·12	+†	+
Fat		1·14 ± 0·88	0·35 ± 0·08	0·20 ± 0.08	0·09 ± 0·09
Brain		0·01 ± 0·00	+	+	+
Lung		0·14 ± 0·02	0·03 ± 0·02	+	+
Muscle		3·60 ± 1·46	0·65 ± 0·32	0·16 ± 0·11	0·12 ± 0·11
Kidney		0·54 ± 0·21	0·16 ± 0·11	+	+
Bile		0·33 ± 0·14	0·06 ± 0·01	+	+
Gastro-intestinal tract		32·74 ± 13·33	7·20 ± 2·07	0·40 ± 0·25	+
Urine		54·81 ± 11·43	88·62 ± 3·03	69·86 ± 22·60	67·71 ± 19·93
Faeces		+	0·03 ± 0·02	3·24 ± 0·86	5·02 ± 0·84
	Recovery (% of dose)...	95·27 ± 0·54	97·63 ± 0·91	73·90 ± 23·35	75·00 ± 21·93

*Values are mean percentages of the dose ± SEM/whole organ or total urine or faeces. Fat was estimated to be 22·1% of body weight.
†Present, but in amounts too small to be estimated (<0·01% of dose/g sample).

Table 7. *Percentage of water-soluble radioactivity (metabolized compound) in tissues and excreta of animals dosed orally with 50 mg [^{14}C]phthalate/kg in corn-oil solution*

Species	Water-soluble radioactivity (% of total radioactivity) derived from					
	Diisooctyl [^{14}C]phthalate at			Butylglycolylbutyl [^{14}C]-phthalate at		
	4 hr	24 hr	4 days	4 hr	24 hr	4 days
Rat	40·9	85·5	96·2	63·8	98·5	99·2
Dog	40·8	52·1	63·5	81·5	94·8	98·3
Pig	30·7	55·8	71·4	72·1	81·7	99·2

again the nature of the ester side chain appears to influence excretion of the ester. It appears that BGBP, because of its comparatively polar side chain, is distributed into fat to a lesser degree and thus is more readily metabolized and excreted.

The observation that pigs required 21 days to excrete the dose of [^{14}C]DIOP completely, and yet displayed less enterohepatic recycling than dogs, is probably explained by the fact that the pig normally has a higher amount of body fat than the dog. The lipophilic DIOP is distributed into body fat and then slowly released, metabolized and excreted without extensive enterohepatic recycling.

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SHORT-TERM ORAL TOXICITY STUDY OF DIETHYL PHTHALATE IN THE RAT

D. BROWN, K. R. BUTTERWORTH, I. F. GAUNT, P. GRASSO and S. D. GANGOLLI

British Industrial Biological Research Association, Woodmansterne Road, Carshalton, Surrey SM5 4DS, England

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Abstract—Groups of 15 male and 15 female rats were given diets containing 0 (control), 0·2, 1·0 or 5·0% diethyl phthalate (DEP) for 16 wk. Consumption of diet containing 5% DEP was associated with a reduction in food intake and in the rate of body-weight gain, and similar effects were seen in the females given 1% DEP. No statistically significant effects on water intake or on the results of the haematological examinations, serum-enzyme levels, urinary cell-excretion rate, renal concentration tests or histological examination were seen in the treated animals. However, there were increases at wk 16 in the relative liver weight of females at all treatment levels and of males fed the highest level. There were also increases in the absolute and relative weights of stomach and small intestine in rats of both sexes; in the females these were statistically significant at all dietary levels at wk 16.

INTRODUCTION

Phthalates are noted for their low acute toxicity (Lawrence, Malik, Turner, Singh & Autian, 1975) and are widely used in industry, particularly as plasticizers. The toxicity of the phthalates has recently come under review since they have been demonstrated in transfusion solutions stored in polyvinyl chloride 'blood-storage bags' (Guess, Jacob & Autian, 1967) and in the tissues of patients after extensive blood transfusions (Jaeger & Rubin, 1970). The leaching of phthalates from haemodialysis equipment was demonstrated and its implication in the production of hepatitis in such patients suggested by Neergard, Nielson, Faurby, Christensen & Nielsen (1971). The danger indicated by these studies is that the phthalates could be administered in this way to debilitated patients who might be expected to be less than normally resistant to such a challenge.

The main use of diethyl phthalate (DEP) as a plasticizer is in the production of cellulose acetate films, in which it may constitute up to 20% of the finished product. In addition it is used extensively as a denaturant for cosmetic alcohol and in hair-spray preparations. It is permitted in the UK as a denaturant for industrial alcohol at a level of 1% for cosmetic use (Statutory Instrument 1952, no. 2230). In the USA it is permitted for a number of food-contact applications, notably as a component of paper and paperboard in contact with aqueous and fatty foods (Sec. 176.170, formerly 121.2526, of the Code of Federal Regulations) and in resinous and polymeric coatings (Sec. 175.320, formerly 121.2569). The percutaneous absorption of some phthalates has been demonstrated (Fassett, 1963), so that the question of systemic intoxication from the cosmetic use of DEP must be considered.

The *in vivo* metabolism of dialkyl phthalates in the rat following oral ingestion has been studied extensively (Albro & Moore, 1974; Albro, Thomas & Fishbein, 1973; Williams & Blanchfield, 1975). The pattern of metabolism is broadly similar for all the members of the homologous series studied, including the dimethyl, di-*n*-butyl and di-*n*-octyl phthalates, and involves hydrolysis to the monoester with subsequent oxidation of the remaining alkyl chain. In all the studies cited above, phthalic acid was found to be a minor metabolite, accounting generally for less than 5% of the total urinary metabolites, although with dimethyl phthalate, phthalic acid accounted for 14% of the total. Recently this work was extended in a study that demonstrated the *in vivo* hydrolysis of various phthalate esters, including DEP, by hepatic and intestinal mucosal preparations from the rat, ferret and baboon (Lake, Phillips, Hodgson, Severn, Gangolli & Lloyd, 1976). These workers found an inverse relationship between the length of the alkyl side chain and the rate of hydrolysis by the intestinal mucosa. The dialkyl phthalates are metabolized by the same route in the human small intestine as in the species described above (B. G. Lake, personal communication 1976).

DEP has been shown to be toxic to cells in culture systems (Calley, Autian & Guess, 1966; Fishbein & Albro, 1972) and to be teratogenic in the chick embryo (Bower, Haberman & Minton, 1970) and rat (Singh, Lawrence & Autian, 1972). In the rat study, DEP was shown to give rise to an increased incidence of resorptions and a dose-dependent increase in skeletal abnormalities, but the doses used were rather high (one third to one tenth of the LD_{50}).

There has recently been some criticism of the methodology used in some of these experiments and of the conclusions reached (Autian, 1973) and a need has been suggested for the investigation of possible cumulative effects of repeated small doses of DEP (Singh *et al.* 1972). The present study was initiated, therefore, to investigate the effect of DEP in a short-term feeding study in the rat.

EXPERIMENTAL

Materials. The DEP, obtained from BP Chemicals Ltd., Epsom, Surrey, conformed to the following specification: density at 20°C, 1·117; boiling point, 296°C; ester content, min. 99%; acidity as phthalic

acid, max 0·01%; water content, max 0·10%; ash, max 0·01%; refractive index, 1·500–1·505.

Animals and diet. Rats of the CD strain (Sprague–Dawley-derived) were obtained from Charles River U.K. Ltd., Margate, Kent, and housed in an animal room maintained at 21 ± 1°C with a relative humidity of 50–60%. They were fed Spratts Laboratory Diet No. 1 and allowed access to water *ad lib.*

Experimental design and conduct

Short-term feeding study. Groups of 15 rats of each sex were given diets containing 0 (control), 0·2, 1·0 or 5·0% DEP for 16 wk. Additional groups of five rats of each sex were fed similar diets for 2 or 6 wk. Body weight, food intake and water intake were measured weekly. At the end of the appropriate feeding period the rats were deprived of food overnight and killed by exsanguination from the aorta under barbiturate anaesthesia. Blood samples were collected from the aorta for haematological examination and at wk 16, for serum analyses.

An autopsy was carried out during which any macroscopic lesions were noted and the brain, pituitary, thyroid, heart, liver, kidney, adrenal glands, spleen, gonads, stomach, small intestine and caecum (with and without its contents) were weighed. Samples of these organs and of salivary glands, trachea, lung, aorta, lymph nodes, thymus, urinary bladder, oesophagus, colon, rectum, pancreas, uterus or prostate and seminal vesicles, skeletal muscle, eye, harderian gland and sciatic nerve were preserved in 10% buffered formalin. Paraffin-wax sections of the tissues were stained with haematoxylin and eosin for histological examination.

Using the blood collected at the post-mortem examination, the haemoglobin concentration and packed cell volume were measured and counts were made of total erythrocytes and total leucocytes. Differential leucocyte counts were performed on blood films prepared at wk 2, 6 and 16 from rats fed the control diet or highest dietary level of DEP. Marrow smears were prepared and stained, but were not examined as there was an absence of haematological effects. The serum collected at wk 16 was analysed for glutamic–pyruvic and glutamic–oxalacetic transaminases and for lactic dehydrogenase.

Urine collected during wk 2, 6 and 13 of the feeding study was examined for cells and other microscopic constituents and semi-quantitative tests were carried out for protein, glucose, ketones, bile and blood. A concentration test was carried out by measuring the volume and specific gravity of urine collected during a 6-hr period of water deprivation and in a 4-hr period commencing after 16 hr without water. At wk 6 and 13, a dilution test was included by making the same measurements on urine produced in a 2-hr period following a water load of 25 ml/kg.

Paired-feeding study. Groups of six rats of each sex were caged individually and fed diet containing either 0 (control) or 5·0% DEP for 112 days. Each control animal was from the same litter as one of the treated rats of the same sex. The treated rats were fed *ad lib.* and the food intake of each was recorded daily. Each control rat was given an amount of food equal to that consumed in the previous 24-hr period by its paired litter-mate. Body weights were measured weekly.

RESULTS

Short-term feeding study

Rats given diets containing DEP showed no changes in behaviour patterns nor other clinical signs of toxicity. Both sexes given 5% DEP in the diet gained significantly less weight than the controls (Table 1). A reduction of body-weight gain was seen also in the female rats fed diet containing 1% DEP. In these groups lower body weights were seen throughout the experiment after the first 24 hr of treatment. A transient but statistically significant effect was apparent in male rats given the 1% dietary level, the body weights of this group being lower than those of controls only from day 6 to 36. Lower body weights were accompanied by a decrease in food consumption (Table 1), which was most evident during the first day of the study. The mean food consump-

Table 1. *Mean body weight and food and water consumption of rats fed DEP at 0–5% in the diet for 16 wk*

Dietary level (%)	Body weight (g) at day				Food consumption (g/rat/day) at day				Mean food consumption (g/rat/day)	Mean water intake (g/rat/day) up to day 112
	0†	27	56	112	1	27	56	112		
Males										
0	125	356	473	599	15·5	27·3	31·3	21·5	24·9	35·2
0·2	125	352	476	617	17·3	29·7	29·5	21·7	25·3	33·4
1·0	124	334*	459	575	13·8	30·0	25·9	21·8	24·7	31·7
5·0	124	271*	356***	461***	3·2*	25·5	23·8	20·2	19·1*	34·4
Females										
0	109	225	283	358	13·7	18·5	21·9	15·2	18·5	31·9
0·2	109	235	286	347	13·9	19·3	17·1	15·8	17·7	27·1
1·0	109	213*	266*	328*	11·4	16·2	19·3	14·7	16·5*	35·5
5·0	108	191***	234***	285***	3·7*	16·6	16·1	14·3	15·1*	28·1

† First day of feeding.

Body weights are means for groups of 15 animals, and food and water consumption figures are means for three cages each of five rats. Values marked with asterisks differ significantly (Student's t test for body weights and ranking method of Wilcoxon (1945) for food consumption) from those of the controls: $^{*}P < 0{\cdot}05$; $^{***}P < 0{\cdot}001$.

Table 2. *Results of haematological studies in rats fed DEP at 0–5% in the diet for 6 or 16 wk*

Dietary level (%)	No. of rats examined	Hb (g/100 ml)	PCV (%)	RBC (10^6/mm^3)	Retics (% of RBC)	Leucocytes Total (10^3/mm^3)	Differential (%) N	E	L	M
				Wk 6						
Male										
0	5	15·5	46	6·71	0·4	5·2	13	1	85	1
1·0	5	16·6	46	7·42	—	4·7	—	—	—	—
5·0	5	17·0	46	8·64***	0·5	4·8	24	0	75	1
Female										
0	5	15·4	46	7·23	0·6	5·6	10	1	89	0
1·0	5	14·9	45	6·89	—	5·8	—	—	—	—
5·0	5	15·5	47	7·24	0·4	7·5	5	0	95	0
				Wk 16						
Male										
0	15	14·8	44	7·45	2·1	5·2	17	1	80	2
0·2	13	15·3	46	7·89	—	6·2	—	—	—	—
1·0	15	15·5	46	7·79	—	5·9	—	—	—	—
5·0	14	15·5	45	7·98	0·6	6·2	16	1	81	2
Female										
0	15	15·5	44	7·15	0·8	3·9	12	1	86	1
0·2	15	15·4	44	7·55	—	4·3	—	—	—	—
1.0	15	14·8	43	7·16	—	3·8	—	—	—	—
5·0	15	15·3	43	7·09	0·8	4·2	14	1	83	2

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

Values are means for the numbers of rats indicated and that marked with asterisks differs significantly ($P < 0·001$ by Student's t test) from the control value.

tion throughout the study was significantly lower than that of controls in rats of both sexes given 5% DEP in the diet and in females given 1%. The mean intakes of DEP over the entire study were approximately 150, 770 and 3160 mg/kg/day in the males of the three test groups and 150, 750 and 3710 mg/kg/day in the females. There were no notable inter-group differences in water consumption at any stage of the study.

The erythrocyte count was significantly higher in male rats given 5% dietary DEP for 6 wk than in the corresponding controls (Table 2). This increase was accompanied by a haemoglobin level higher than, but not differing significantly from, the control value, but neither of these values was raised at wk 2 or 16. Consumption of diets containing DEP did not lead to significant changes in the levels of any of the serum enzymes studied.

There was no significant increase in urinary cell excretion (Table 3) in any of the DEP-treated groups and the male rats given 5% DEP excreted significantly fewer cells at wk 13. At wk 2, the females fed diet containing 5% DEP produced a slightly more concentrated urine after a 6-hr period of water deprivation than did the controls and a similar effect was observed in the male 5% group at wk 6. However, there were no statistically significant differences between test and control groups after a more prolonged period of water deprivation. During the 2-hr urine-dilution test at wk 6, male rats receiving 5% dietary DEP excreted a significantly larger volume of more dilute urine than did the controls. Conversely, female animals in the 5% group excreted a significantly smaller volume of urine. At wk 13, the urine of male rats given 5% dietary DEP was significantly more concentrated than that of the controls.

The only gross abnormality found at autopsy was a unilaterally small testis in one rat which had received DEP at 0·2% in the diet. The histological appearance of this testis was normal. Weights of the brain, heart, spleen and kidneys were low in males and/or females fed 5% DEP for 16 wk, the differences from controls being statistically significant. Female gonads and the heart, spleen and kidneys in males showed similar reductions at the earlier stages. Stomach weights of rats receiving DEP increased at various times throughout the study, but were statistically significant only at wk 2 in males receiving 5% DEP and at wk 2 and 16 in females given the same dietary level. The weight of the full caecum showed a statistically significant rise in female rats given 5% DEP in the diet for 16 wk. At wk 16, no significant changes in the absolute weights of any organs were observed at dietary levels below 5%, although in the groups on 1% DEP values significantly ($P < 0·05$) higher than the controls were recorded for the male kidneys at wk 2 and female gonads at wk 6 and a lower pituitary weight ($P < 0·05$) was found in females at wk 2. Terminal body weights of all rats given 5% dietary DEP were reduced significantly, as was the body weight of females receiving 1% dietary DEP for 16 wk (Table 4).

The relative weights (Table 4) of the brain, liver, stomach, small intestine and full caecum were higher in both sexes receiving 5% DEP than in the controls. In addition, the relative testis weight was raised con-

Table 3. *Results of urinary cell excretion and concentration test in rats fed DEP at 0–5% in the diet for 2, 6 or 13 wk*

Sex and dietary level (%)	No. of rats	Cell excretion (10^3/hr)	Concentration test: Specific gravity 6 hr	Concentration test: Specific gravity 20 hr	Concentration test: Volume (ml) 0–6 hr	Concentration test: Volume (ml) 16–20 hr	Dilution test (0–2 hr): Specific gravity	Dilution test (0–2 hr): Volume (ml)
				Wk 2				
Male								
0	15		1·029	1·068	2·7	0·5	—	—
1·0	5		1·032	1·079	2·3	0·3	—	—
5·0	5		1·038	1·057	2·8	1·2	—	—
Female								
0	15		1·019	1·068	2·2	0·6	—	—
1·0	5		1·014	1·069	2·0	0·6	—	—
5·0	5		1·037**	1·067	4·1	0·8	—	—
				Wk 6				
Male								
0	15	0·8	1·018	1·068	5·0	1·1	1·045	1·4
1·0	15	0·9	1·023	1·068	3·7	0·9	1·021	2·1
5·0	15	1·4	1·040*	1·043	4·2	1·4	1·020	4·5*
Female								
0	15	0·9	1·019	1·066	3·1	0·6	1·003	8·0
1·0	15	0·8	1·013	1·067	1·7	0·7	1·004	7·2
5·0	15	2·0	1·028	1·058	2·5	0·6	1·007	5·5**
				Wk 13				
Male								
0	12	0·6	1·022	1·058	4·6	0·7	1·004	10·9
0·2	12	1·6	1·023	1·061	3·8	0·8	1·005	9·1
1·0	11	0·4	1·025	1·071	3·8	0·9	1·005	10·4
5.0	11	0·1***	1·033	1·049	3·3	1·7	1·012*	8·1
Female								
0	12	0·4	1·029	1·052	1·9	0·6	1·004	6·8
0·2	12	0·2	1·026	1·077	2·8	0·4	1·004	7·8
1·0	12	0·7	1·027	1·077	1·9	0·3	1·005	7·2
5·0	12	0·2	1·034	1·074	2·7	0·5	1·005	6·3

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

sistently in rats given 5% DEP and at wk 16 the relative kidney weights of males and females receiving the 5% level were markedly higher than those of the controls. There were some similar findings among the relative weights of other organs, notably the liver, stomach and small intestine, at the lower dose levels.

Some fatty degeneration and slight vacuolation of the liver, as well as pyelonephritis and lymphocytic infiltration of the kidney were observed. These effects were not dose related despite the consistent effect of DEP on the weight of these organs. The incidences of other microscopic lesions were similar in the test and control groups.

Paired-feeding study

Animals given 5% DEP in the diet showed a more pronounced loss of weight over the first day of the experiment than pair-fed controls (Table 5). Similarly the rate of weight gain was lower in test animals than in those receiving the control diet, although in this study the difference was not statistically significant until the final week of the study. The total food consumed by the animals given 5% DEP in the diet was greater than that consumed by the control animals.

DISCUSSION

A more marked depression of food intake was observed at the beginning than at the end of the study, a pattern often associated with an unpalatable diet. DEP has been shown to be irritant to mucous membranes (Sax, 1975), but unpalatability of the diet was not solely responsible for the lower body weights since in the paired-feeding study the test rats gained significantly less weight than controls. This suggests that in addition to its effect on food consumption, DEP also affects the efficiency of utilization of the food.

A pattern of reduction in absolute organ weight with an increase in the relative value was seen in the brain, heart, spleen, kidneys, adrenal glands, gonads and pituitary. On the other hand, the pattern of organ weights seen with the liver and various parts of the gastro-intestinal tract was different, with normal or slightly increased values for the absolute weights and consistent increases when these weights were expressed relative to body weight. It is likely that many of these changes were a result of an alteration in the normal growth rate of the rats since other authors have observed a similar pattern of changes in rats maintained under conditions that retarded growth (Feron, de Groot, Spanjers & Til, 1973) or fed compounds that caused a reduction in the growth rate (Carpanini, Gaunt, Hardy, Gangolli, Butterworth & Lloyd, 1978; Gaunt, Sharratt, Grasso, Lansdown

Table 4. *Relative organ weights of rats fed 0–5% DEP in the diet for 2, 6 or 16 wk*

Sex and dietary level (%)	No. of rats	Relative organ weights (g/100 g body weight) Brain	Heart	Liver	Spleen	Kidneys	Stomach	Small intestine	Caecum Empty	Caecum Full	Adrenals†	Gonads‡	Pituitary†	Thyroid†	Terminal body weight (g)
Wk 2															
Male															
0	5	0·97	0·46	3·37	0·30	0·93	0·55	3·66	0·42	1·79	18·4	1·07	3·9	6·8	194
1·0	5	0·99	0·46	3.67*	0·28	0·99	0·59	3·67	0·46	1·51	20·3	1·05	3·0	7·3	193
5·0	5	1·23*	0·44	4·78***	0·30	1·04*	0·84**	3·97	0·53	2·24	21·5	1·32**	3·7	7·5	149***
Female															
0	5	1·15	0·42	3·63	0·27	1·00	0·83	3·35	0·44	1·71	33·4	61	5·4	7·8	157
1·0	5	1·20	0·41	3·56	0·28	0·99	0·64	3·29	0·48	1·65	27·8	70	5·1	9·9	149
5·0	5	1·31*	0·42	4·81***	0·29	1·06	0·86	3·79**	0·49	2·14	26·1	57	4·7	10·9	134***
Wk 6															
Male															
0	5	0·55	0·33	2·56	0·18	0·76	0·39	1·89	0·27	0·74	16·2	0·86	2·9	4·8	398
1·0	5	0·55	0·34	2·94*	0·21	0·75	0·40	2·10	0·30	0·77	13·9	0·94	2·6	4·8	385
5·0	5	0·76**	0·35	3·41**	0·22	0·81	0·52*	2·57***	0·36*	1·15*	17·5	1·23**	2·8	6·5**	274***
Female															
0	5	0·80	0·40	2·69	0·26	0·74	0·46	2·76	0·40	1·01	28·6	55	4·2	5·5	237
1·0	5	0·84	0·41	2·93	0·26	0·70	0·48	2·58	0.45	1·34	29·9	60	4·2	6·4	231
5·0	5	0·97**	0·41	3·57***	0·23	0·81	0·59***	2·78	0·42	1·50**	30·9	49	3·7	6·6	199**
Wk 16															
Male															
0	15	0·39	0·27	2·22	0·15	0·57	0·29	1·54	0·21	0·60	9·6	0·66	2·1	3·5	568
0·2	15	0·39	0·29	2·16	0·13	0·58	0·29	1·53	0·23	0·60	9·1	0·64	2·0	3·2	585
1·0	15	0·39	0·27	2·29	0·14	0·57	0·32*	1·57	0·24	0·71	9·3	0·66	2·2	3·5	559
5·0	15	0·50***	0·31**	2·95***	0·14	0·67***	0·41***	1·93***	0·24	0·89***	11·2*	0·85***	2·5***	4·1**	438***
Female															
0	15	0·65	0·31	2·17	0·16	0·62	0·35	1·99	0·27	0·77	19·7	26	4·7	4·9	330
0·2	15	0·64	0·32	2·31*	0·16	0·62	0·40***	2·23**	0·30*	0·86	19·1	27	4·5	5·0	328
1·0	15	0·68	0·33	2·35**	0·17	0·64	0·41***	2·26*	0·29	0·89	20·2	28	4·7	5·2	304*
5·0	15	0·78***	0·32	2·84***	0·19**	0·69***	0·51***	2·47***	0·34***	1·43***	22·1	28	4·4	5·8	267***

† Relative weights of these organs are expressed in mg/100 g body weight.
‡ Relative weights of female gonads are expressed in mg/100 g body weight.
Values are the means for the numbers of rats shown and those marked with asterisks differ significantly (Student's *t* test) from the control values: * $P < 0·05$; ** $P < 0·01$; *** $P < 0·001$.

Table 5. *Body weight and food consumption of rats fed DEP at 5% in the diet and of pair-fed controls*

Dietary level (%)	Body weight (g) at day						Weight gain (g)	Total food consumed to day 112 (g/rat)
	0†	1	20	42	70	112		
Male								
0	97	94	208	327	450	540	446	2430
5	91	83**	206	307	423	498*	415*	2464
Female								
0	85	85	160	208	262	300	215	1724
5	88	78*	159	198	247	272*	194*	1821

† First day of feeding.

Values are means of groups of five males and six females and those marked with an asterisk differ significantly (ranking test of Wilcoxon, 1945) from the control values: $*P < 0.05$; $**P < 0.01$.

& Gangolli, 1974). However, in some groups increases in relative weights were observed in the absence of a reduction in growth rate. Thus, while the increases in the relative weight of the stomach and small intestine of females were associated with reduced growth rates in groups fed 5 or 1% DEP, similar weight changes occurred at the 0·2% level although the body weight was unaffected. Some other factor was clearly responsible for these alterations at the lower dose level. One possibility is the irritant nature of DEP (Calley *et al*., 1966; Sax, 1975) although there was no histological evidence to support this suggestion. Another possibility arises from the finding (Rowland, 1974) that the metabolism of another phthalate ester (di-(2-ethylhexyl) phthalate) by intestinal contents was increased in rats previously exposed to the ester. This increased metabolism was attributed to the induction of an esterase in the mucosal cells rather than to the selective growth of a microflora with esterase activity. Since the metabolism of DEP by the gastro-intestinal mucosa of various species, including the rat, has been demonstrated (Lake *et al*. 1976), the observed increase in relative weight of parts of the gastro-intestinal tract might have been due, at least in part, to esterase induction in the mucosal cells. In that case the increased weights could have been a reflection of the use of high doses and the consequent excessive load on the metabolic capacity of the mucosa. As such, they would not be considered to be a toxic effect. However, the low weights of the gastro-intestinal tract in the females of the two lower dose levels were probably due to the fact that the pattern of growth of the stomach and small intestine of the female controls was not typical of that seen in recent studies with rats of the same strain. For example, there was no change in small intestine weight in these controls between wk 6 and 16 and only a very small increase in stomach weight, although the body weight increased by 40%. Consequently the weights of stomach and small intestine were lower than would have been expected at the termination of the study. If the more typical values for these organs in untreated rats are compared with the weights in the animals given the 0·2 and 1% levels in this study, little or no difference is observed. It is probable, therefore, that the changes seen in these groups were due to comparison with an inappropriate control and were not due to treatment. Furthermore, the range of the control weights was almost half that seen in the test values and this may have contributed considerably to the high degree of statistical significance calculated.

Caecal enlargement can be produced by a wide range of materials, including food colourings, detergents, neomycin and modified starches. Its toxicological significance is not understood, but it has been suggested by Butterworth, Lake, Mason & Rowland (1975) that it may be a work hypertrophy due to an increase in the bulk of the intestinal contents. Leegwater, de Groot & Van Kalmthout-Kuyper (1974) have suggested that caecal enlargement may be due to an increase in the osmotic activity of the intestinal contents causing the retention of water and an increase in the bulk of the gut contents. A decrease in the numbers of bacteria that break down dietary cellulose in the rat (Conrad, Watts, Iacono, Kraybill & Friedemann, 1958) could lead to such a change in osmotic activity. Administration of di-(2-ethylhexyl) phthalate to rats has been shown to lead to a slight decrease in the total numbers of bacteria in the stomach and small intestine and to a marked alteration in the bacterial spectrum, with almost complete loss of aerobes (Rowland, 1974). These changes could significantly affect the composition and therefore the osmotic properties of material reaching the caecum. It is perhaps noteworthy that in the current study the bulk of the increase in caecal weight was due to an increase in the weight of the contents.

Despite the changes in liver weight there was no histological evidence of damage. The significance of such hepatic enlargement in the absence of histological change is a contentious issue and much effort has been devoted to the elucidation of the mechanisms involved (Schulte-Hermann, 1974). Some workers have suggested that in order to evaluate the toxicological significance of liver enlargement it is necessary to examine the fine structure and to determine the levels of marker enzymes in various subcellular fractions (Feuer, Goldberg & Le Pelley, 1965; Grasso, Wright, Gangolli & Hendy, 1974; Weil, 1970). Cytopathological changes have been demonstrated in enlarged but histologically normal livers, and the conclusion has been drawn that in the case of work hypertrophy there is a stimulation of processing-enzyme activity and an increase in the amount of

smooth endoplasmic reticulum, whereas damaged livers show a reduction in the activities of aniline hydroxylase and some other processing enzymes and in glucose-6-phosphatase activity. In addition, free ribosomes are sometimes observed.

This aspect of the biological activity of DEP has been studied by B. G. Lake (personal communication 1976) following the oral administration of DEP in corn oil to male Sprague–Dawley rats at a dosage of 1140 mg/kg/day for 14 days. This dosage, which was of the same order as the intake of DEP observed at the 1% dietary level in the first 2 wk of the present study, produced some liver enlargement, evidence of induction of hepatic processing enzymes (in particular an increase in the activity of ethylmorphine *N*-demethylase and cytochrome *P*-450) and no marked loss of either aniline-hydroxylase or glucose-6-phosphatase activity. However, there was no increase in smooth endoplasmic reticulum, as shown by an essentially unchanged value for microsomal protein, and there was a decrease in the activity of alcohol dehydrogenase. Overall, this pattern of change is most consistent with a functional hypertrophy of the liver and, on this basis, it is likely that the liver enlargement reported in this paper was the result of such hypertrophy. On this evidence there is no reason to assume that the enlarged liver represents an adverse response to DEP. However, in considering the possible effects of prolonged DEP consumption in man, it is relevant that continued stimulation of processing enzymes has been shown to lead to the development of vitamin D-resistant rickets and macrocytic anaemias related to folate deficiency (Kruse, 1968; Richens, 1972).

The significance of an enlarged, histologically normal kidney is equally open to debate. At the termination of the present study the relative kidney weight was increased in both males and females receiving 5% DEP in the diet, although there was no apparent loss of renal competence or increased incidence of histological change. It may be concluded from this study, therefore, that dietary levels of 1% DEP or less were without adverse effect on the kidney, although in the light of present knowledge the increased relative kidney weight seen at the 5% level must be considered an untoward effect of treatment. Kidney enlargement similar to that observed in the present study has also been observed in rats receiving di-(2-ethylhexyl) phthalate for 6 months (Gray, Butterworth, Gaunt, Grasso & Gangolli, 1977) and dialkyl 79 phthalate for 90 days (Gaunt, Colley, Grasso, Lansdown & Gangolli, 1968) and it was concluded that long-term exposure to high levels of these phthalates could result in kidney damage.

In the present study consumption of DEP at a dietary level of 5% produced a number of untoward effects. At the lower levels the only consistent change that was thought to be due to treatment was liver enlargement, and it has been argued that this was possibly due to a work hypertrophy. In addition, however, there was a reduced growth rate in female rats given 1% DEP, which may be considered to be an adverse effect. On this basis, a no-untoward-effect level of 0·2% (about 150 mg/kg/day) could be postulated. However, even at this level there was some indication of an increase in liver weight and the possible consequences to man of prolonged induction of processing enzymes cannot, therefore, be altogether discounted.

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ALTERATION OF HEPATIC DETOXICATION ENZYME ACTIVITY BY DIETARY ARSENIC TRIOXIDE

D. J. WAGSTAFF
Epidemiology Unit, Bureau of Foods, Food and Drug Administration, Washington, DC 20204, USA

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Abstract—The effects of dietary arsenic trioxide (As_2O_3) on liver detoxication enzymes were studied. Enzyme activity was monitored by measuring hexobarbitone sleeping time, oxidative cleavage of *O*-ethyl-*O*-*p*-nitrophenyl phenylphosphonothioate (EPN) and *O*-demethylation of *p*-nitroanisole, in rats fed diets containing 0, 100, 500, 1000, 2000 or 5000 ppm As_2O_3 for 15 days. Hexobarbitone sleeping time was decreased in rats given 100 ppm As_2O_3 or more; the other enzyme responses were stimulated considerably in rats given 1000 ppm As_2O_3 and most of the rats given 2000 or 5000 ppm died. The capacity of the enzyme system to be stimulated by dietary sodium phenobarbitone was not impaired by As_2O_3.

INTRODUCTION

Arsenic compounds have long been used as therapeutic agents and pesticides. The usual, but not invariable, result of exposure to compounds of arsenic is inhibition of enzymes and metabolic processes (Webb, 1966). Extensive studies have been made of the effects of arsenic on enzyme processes involving energy production. Considerably less experimental work has been done on the effects of arsenic on the activity of detoxication enzymes. If detoxication processes were impaired, the toxicity of numerous substances would be enhanced. Although there is some evidence that under certain conditions arsenic stimulates enzyme detoxication processes in micro-organisms (Legge, 1954; Legge & Turner, 1954; Turner & Legge, 1954), little is known about this effect in mammals. Ribeiro (1971) reported that hexobarbitone anaesthesia time was reduced in mice pretreated with As_2O_3 but that rates of hexobarbitone oxidation or *N*-demethylation of aminopyrine were not altered in liver tissue from these same mice.

The purpose of the present two-part study was to determine whether As_2O_3 fed to rats impairs hepatic detoxication enzyme activity and whether, under some circumstances, there is enzyme enhancement. In Experiment 1, the effects of various levels of As_2O_3 were studied. Experiment 2 was a study of the interactions of As_2O_3 with a drug that stimulates microsomal-enzyme activity, namely, sodium phenobarbitone (PB).

EXPERIMENTAL

Female Holtzman rats (five per group) weighing 100–110 g at the start of each study, were used. Each rat was housed in a separate, suspended stainless-steel cage. The rats were fed a semi-purified basal diet (a USP XII vitamin A test diet fortified with 0·69 ppm vitamin A acetate) *ad lib.* daily for 15 days. In experiment 1, primary standard-grade As_2O_3 (Fisher Scientific Co., Pittsburgh, PA) was mixed into the basal diet at 0, 100, 500, 1000, 2000 and 5000 ppm. The experiment was partially replicated three times. Two rats fed 1000 ppm died by day 7 and one control and two rats fed 100 ppm died during hexobarbitone anaesthesia so that there were 14 control rats, 8 at 100 ppm, 15 at 500 ppm and 13 at 1000 ppm. In experiment 2, a completely randomized design with factorially arranged treatments was used. Six groups of rats were fed diets containing: (1) basal diet alone, (2) 500 ppm As_2O_3, (3) 1000 ppm As_2O_3, (4) 500 ppm PB, (5) 500 ppm As_2O_3 and 500 ppm PB, and (6) 1000 ppm As_2O_3 and 500 ppm PB.

In both experiments, the activity of liver microsomal enzymes was assayed by an *in vivo* method (hexobarbitone anaesthesia) and by two *in vitro* methods (*O*-ethyl-*O*-*p*-nitrophenyl phenylphosphonothionate (EPN) detoxication and *O*-demethylation of *p*-nitroanisole). After 10 days of feeding, each rat was injected ip with 90 mg sodium hexobarbitone/kg body weight and the period of inactivation of the righting reflex was recorded as hexobarbitone sleeping time. A decrease in sleeping time was accepted as presumptive evidence of liver microsomal-enzyme stimulation (Axelrod, 1965). After 15 days of feeding, each rat was killed with ethyl ether, the liver was excised, and a 1 g portion was ground in a glass-Teflon tissue homogenizer with 4 ml cold 1·15% KCl. The homogenate was centrifuged at 9000 *g* for 15 min at 5°C. The supernatant fluid was assayed by the method of Kinoshita, Frawley & DuBois (1966) for oxidative cleavage of the insecticide EPN and *O*-demethylation of *p*-nitroanisole (*O*-demethylase). The product in both assays was *p*-nitrophenol.

RESULTS

Experiment 1: Effects of dietary arsenic trioxide

The effects of different dietary levels of As_2O_3 are summarized in Fig. 1. Microsomal-enzyme activity was not decreased at any level of As_2O_3. On the contrary, at the 1000 ppm feeding level enzyme activity increased significantly, as measured by all of the methods used. Liver weight also increased in the group of rats fed the 1000 ppm level. The most sensitive indicator of stimulated enzyme activity was hexo-

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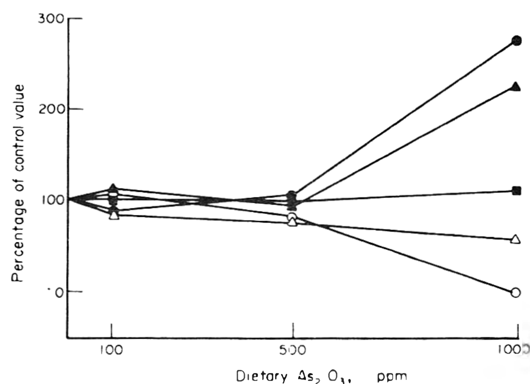

Fig. 1. Effects of dietary As_2O_3 of body-weight gain (○), liver weight (■) and liver detoxication-enzyme activities as indicated by hexobarbitone sleeping time (△), EPN detoxication (▲) and *O*-demethylation of *p*-nitroanisole (●). All values at 1000 ppm differed significantly from control values (Student's *t* test, $P < 0.05$). At 500 ppm, body-weight gain and hexobarbitone sleeping time were significantly altered. At 100 ppm, only hexobarbitone sleeping time was significantly different from the control value.

barbitone sleeping time, which was significantly altered at levels of As_2O_3 of 100 ppm and above.

Arsenic toxicity was dose-related. There were no arsenic-related deaths among rats fed the 0, 100 or 500 ppm level of As_2O_3, but among the 15 animals fed 1000 ppm there were two deaths. Four of the five rats fed the 2000 ppm level died, and all five rats fed the 5000 ppm level died. These deaths occurred during the first 7 days of each of the feeding periods. Body-weight gains were depressed by levels of As_2O_3 above 100 ppm. Rats fed a diet containing 500 ppm As_2O_3 gained weight at only 81% of the control rate. Survivors among animals fed the 1000 ppm level lost 6% of their starting body weight during the 15-day feeding period. Feed consumption was not significantly altered at 100 ppm but at 500 and 1000 ppm As_2O_3 it dropped to 80 and 73% of the control, respectively. In addition to depressed body-weight gain and depressed feed consumption, the signs of intoxication included roughened hair, diarrhoea and reduced physical activity.

Experiment 2: Arsenic trioxide-phenobarbitone interaction

Results of the As_2O_3–PB experiment are summarized in Table 1. When the As_2O_3 was fed alone (groups 2 and 3), the effects on enzyme activity, liver weight, growth and feed consumption were similar to those observed in experiment 1 but there were no deaths in this experiment. As expected, PB in the diet caused a significant increase in enzyme activity and liver weight (group 4, Table 1). For example, hexobarbitone anaesthesia was almost obliterated in the PB-treated rats. When the As_2O_3 and PB were fed in the same diet (groups 5 and 6 of Table 1) the effect on enzyme activity and liver weight was greater than that produced by either substance alone. However, as seen from the interaction term in the analysis of variance (Table 1), the increases in enzyme activity, liver weight and feed consumption due to the combination of As_2O_3 and PB were not significantly greater than the sum of the effects of these agents fed singly. On the other hand, PB partially counteracted the weight-depressant effects of arsenic. PB also improved the appearance and physical activity of arsenical-fed rats. This protective effect of PB occurred in spite of increased feed consumption and consequently increased arsenic dosage (compare groups 3, 5 and 6 of Table 1).

DISCUSSION

The results of this study provide evidence that As_2O_3 fed to rats does not impair activity of hepatic detoxication enzymes and that in some circumstances there is moderate enzyme induction. The same conclusions can be reached for the effects of As_2O_3 in rats simultaneously fed PB. Therefore, it is probable that the general results observed may apply in a variety of situations.

Knowledge about the effects of As_2O_3 on detoxication processes contributes to the background information essential in establishing allowable environmental levels of arsenic and developing more effective therapeutic procedures for arsenic intoxication. However, unlimited extrapolation of the data in this report would be unwise because of the wide variety in the chemical, physical and biological properties of the many kinds of arsenic compounds.

The mechanism by which As_2O_3 affects enzyme activity can only be conjectured. A number of enzymes are known to be stimulated *in vitro* by arsenic under certain conditions (Webb, 1966). In the intact animal, however, the arsenic compound may act either directly on the enzyme molecule or indirectly by one of several processes. Most substances observed to have a significant effect on microsomal-enzyme activity are fat-soluble to some extent. However, it has been reported that some ionic substances react directly with the microsomal membrane (Kubinski & Kasper, 1971). It is not known whether a specific reaction of arsenic with this membrane takes place. There is evidence that trivalent arsenic is transformed by different species to the pentavalent form (Bencko, Benes & Cikrt, 1976) and that significant amounts of an organic arsenic metabolite are produced (Lakso & Peoples, 1975). Microsomal enzymes may be involved in some such processes. Thus arsenic may stimulate its own detoxication by microsomal enzymes.

On the other hand, enzyme enhancement is possibly a non-specific response to stress in an intoxicated animal. The depressed growth of the rats in the group with the greatest level of enzyme enhancement (1000 ppm As_2O_3) apparently supports this theory. However, two factors indicate that a general stress response is probably not the full explanation for enzyme stimulation. First, hexobarbitone anaesthesia was significantly altered in some rats that gained weight at control rates (those rats fed 100 ppm As_2O_3). Secondly, the enzyme-enhancing capacity was not reduced by diminishing the severity of arsenic intoxication by concurrent feeding of PB. The question of mechanism is further complicated by the possibility, albeit remote, that a contaminant of the As_2O_3 formulation could have affected enzyme activity.

Regardless of the mechanism, it is evident that

Table 1. *Effects of dietary As_2O_3 and sodium phenobarbitone (PB) on activities of liver microsomal enzymes, feed consumption, growth and liver weight in rats in experiment II.*

Group no.	Dietary additives: As_2O_3 (ppm)	Dietary additives: PB (ppm)	Body weight gain or loss† (% of starting weight)	Feed eaten† (% of starting weight)	Liver weight† (% of body weight)	Microsomal enzyme assays: Hexobarbital sleep time† (min)	Microsomal enzyme assays: EPN detoxication† (μmol PNP/g liver/hr)	Microsomal enzyme assays: *O*-Demethylase† (μmol PNP/g liver/hr)
1	0	0	50 ± 4	185 ± 8	5·4 ± 0·1	70 ± 6	0·14 ± 0·01	0·46 ± 0·06
2	500	0	47 ± 3	174 ± 12	5·2 ± 0·2	55 ± 5	0·13 ± 0·02	0·45 ± 0·05
3	1000	0	−11 ± 7	121 ± 17	5·8 ± 0·2	51 ± 20	0·32 ± 0·06	1·08 ± 0·23
4	0	500	54 ± 4	196 ± 6	6·1 ± 0·3	< 1	0·78 ± 0·09	1·06 ± 0·20
5	500	500	47 ± 2	175 ± 2	6·7 ± 0·2	< 1	0·94 ± 0·09	1·68 ± 0·24
6	1000	500	21 ± 6	160 ± 10	7·3 ± 0·4	< 1	1·31 ± 0·11	2·19 ± 0·19

Source of variance	**Degrees of freedom**	**Analyses of variance F values**: Body weight	Feed eaten	Liver weight	Hexobarbital sleep time	EPN detoxication	*O*-Demethylase
As	2	65·411**	12·332*	5·426*	—	12·612**	12·299**
PB	1	11·459**	4·227	34·310**	—	178·000**	44·919**
AsxPB	2	7·327**	1·857	1·147	—	2·736	1·772

PNP = *p*-Nitrophenol

*Significant at $P < 0{\cdot}05$.
**Significant at $P < 0{\cdot}01$.
†All values are means ± SEM.

maintaining detoxication capacity has a high priority among bodily functions during times of limited body growth. For example, even though the rats fed As_2O_3 at 1000 ppm lost weight, their microsomal-enzyme activity was above control values. Also, the 1000 ppm level of As_2O_3 seems not to impair the capacity of microsomal enzymes to be stimulated by PB.

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PRODUCTION OF AFLATOXINS AND OTHER FLUORESCENT METABOLITES BY STRAINS OF *ASPERGILLUS FLAVUS* ISOLATED FROM STAPLE FOODS AND THEIR TOXICITY TO RATS

S. ANGSUBHAKORN
Department of Pathobiology, Faculty of Science, Mahidol University,

N. BHAMARAPRAVATI
Department of Pathology, Faculty of Medicine, Ramathibodi Hospital, Mahidol University,

K. ROMRUEN
Faculty of Graduate Studies, Mahidol University

and

L. PHIENPICHIT, W. THAMAVIT and S. SAHAPHONG
Department of Pathobiology, Faculty of Science, Mahidol University, Bangkok, Thailand

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Abstract—Forty strains of *Aspergillus flavus* isolated from food marketed in Thailand between 1967 and 1969 were re-isolated for toxicological characterization. Approximately 92% produced aflatoxin B_1 or aflatoxins B_1 and B_2. In acute toxicity tests in weanling male Wistar rats, 87·5% of the orally intubated chloroform extracts (CEX) of the isolates caused at least one death in test groups of five rats, compared with 38% of the ip-administered petroleum ether-insoluble (PEI) fractions. Twenty-four unidentified compounds were produced in varying amounts by eighteen strains of *A. flavus*. These compounds, detected as blue and green fluorescent spots on thin-layer chromatograms, may have modified the toxic effect of the aflatoxins. The histopathological changes caused by the CEX and PEI fractions were similar, the main ones being haemorrhagic necrosis of the liver and kidney, congestion of pulmonary vessels and hypercellularity of the alveolar septa of the lung, hydropic degeneration of the cardiac muscle and lymphocytic depletion of the spleen.

INTRODUCTION

The fungal metabolites known as aflatoxins have been produced on several agricultural commodities by strains of *Aspergillus flavus* and *A. parasiticus*, and the wide distribution of aflatoxins in foods has led to concern about their potential hazard to man (Shank, Wogan & Gibson, 1972). Because of our interest in the toxicological characterization of fungi maintained in the Department of Pathobiology, Faculty of Science, 40 strains of *A. flavus* originally collected from foods marketed in Thailand between 1967 and 1969 (Shank *et al.* 1972) and kept in soil tubes for another 2 yr were re-isolated and screened for the production of aflatoxins and their toxic effects.

Hesseltine, Shotwell, Ellis & Stubblefield (1966) reported the production of aflatoxins on rice, wheat, corn, soya beans and sorghum, and concluded that rice was the best substrate for aflatoxin B_1 production whereas wheat was the best for aflatoxin G_1. A large quantity of aflatoxin was produced on polished rice by Shotwell, Hesseltine, Stubblefield & Sorenson (1966), and this was the substrate chosen for the present study.

EXPERIMENTAL

Preparation of fungi. Forty strains of *A. flavus*, kept in soil tubes for 2 yr, were re-isolated and purified on Potato Dextrose Agar (Difco Laboratories, Detroit, MI, USA). Isolates were grown for 14 days on 200 g autoclaved glutinous rice in 2·8-litre Fernbach flasks at 27°C. Uninoculated flasks served as controls. The fungal cultures were extracted using 750 ml chloroform (analytical grade) in a 1-gallon Waring blender, and the chloroform extracts were evaporated in a rotary evaporator. Residues from four flasks (800 g substrate) were collected for biological testing and designated the chloroform extract (CEX) fraction. The CEX from another four flasks was then partitioned against 20 vols petroleum ether (b.p. 40–70°C), kept overnight in the refrigerator, centrifuged, and evaporated to dryness in a stream of nitrogen, to give the petroleum ether-insoluble (PEI) fraction.

Analysis of aflatoxins. The identity and quantity of aflatoxins in the PEI fraction were determined by thin-layer chromatography (TLC) with or without clean-up procedures. For the clean-up steps, a modification of the Eppley (1966) method was used: the PEI fraction was dissolved in a minimal amount of chloroform, the column was eluted with hexane followed by diethyl ether and then the aflatoxins were eluted from the column with chloroform–methanol (97:3, v/v) and evaporated to dryness. TLC plates (silica gel, Kieselgel, GHR; 0·25 mm thick) were spotted both with the extract samples and with a benzene

solution of authentic aflatoxin standards (Southern Utilization Research and Development Laboratories, US Department of Agriculture, New Orleans, LA). The plates were then developed in chloroform–acetone (95:5, v/v) in an equilibrated chamber. Aflatoxins were identified by their R_f values and their blue or green fluorescence under long-wave ultraviolet light (Chromatovue, Ultra-Violet Products, Inc., San Gabriel, CA, USA) by visual comparison with the standards.

Biological tests. Acute toxicity tests were conducted on both CEX and PEI fractions. Altogether 460 weanling male Wistar rats of our stock colony were used, each test group consisting of five animals. The PEI fractions were dissolved in dimethylsulphoxide (BDH Chemicals Ltd., Poole, Dorset, England), and were given ip, whereas the CEX fractions were administered by stomach tube. All of the PEI and CEX doses were equivalent to 50 g of the original rice sample/animal. Control rats were given only dimethylsulphoxide ip or rice extract orally. Survivors and control animals were killed after 7 days and all tissues were fixed in 10% buffered formalin for histopathological examination.

RESULTS

Production of aflatoxin

Of the 40 strains of *A. flavus* studied, 37 (approximately 92%) produced aflatoxins. The amount of PEI fraction extracted from 800 g glutinous rice (Table 1) varied from 37 to 497 mg (nos 343440 and 684639, respectively), while the total content of aflatoxins B_1 and B_2 in the crude PEI fraction varied from 0 to the 2784 μg found in no. 134614. Three strains produced no detectable aflatoxins, 11 strains produced only aflatoxin B_1 and 26 strains produced both B_1 and B_2. Neither aflatoxin G_1 nor aflatoxin G_2 was detected in this group of *A. flavus* isolates, but 18 unidentified blue and green fluorescent spots with various R_F values were observed on the chromatograms of 18 of the isolates (Tables 2 & 3). The greatest quantities of aflatoxin, 2780, 2030 and 1900 μg total aflatoxin B_1 and B_2, were produced by strains no. 134614, 473474 and 684639, respectively.

Biological tests

Rats dying during the experiment lost on average 5–10 g body weight and the survivors gained 5–10 g by the end of 7 days. The overall toxicity of the PEI fractions was lower than that of the CEX fractions, PEI fractions of only 15 strains being associated with at least one death in the test group compared with 35 strains in the case of the CEX fractions (Table 4). The histopathological lesions produced by the CEX and PEI fractions were comparable. In the surviving rats, the livers generally exhibited congestion and fatty degeneration, with bile-duct proliferation in the portal areas. In rats dying before the end of 1 wk, the liver always showed haemorrhagic necrosis, and necrosis was apparent also in the epithelial cells of the proximal convoluted tubules of the kidney. The lung parenchyma showed congestion of the pulmonary capillaries and hypercellularity of the alveolar septa, there was hydropic degeneration of the cardiac muscular fibres, and the spleen was congested and showed lymphocyte depletion.

Table 1. *Aflatoxins produced by strains of* Aspergillus flavus *and determined by thin-layer chromatography of the petroleum ether-insoluble fraction of a chloroform extract of the culture*

A. flavus strain no.	Amount of toxin formed/800 g rice substrate			
	Crude toxin (mg)	Aflatoxins (μg)		
		B_1	B_2	Total
115602	125	0	0	0
154689	129	0	0	0
283455	206	0	0	0
075029	65	0·3	0	0·3
453405	62	0·3	0	0·3
363465	228	1·1	0	1·1
333446	99	0·4	0	0·4
373472	117	0·5	0	0·5
393434	211	1·0	0	1·0
463464	98	0·4	0	0·4
183479	156	3·1	0	3·1
065061	170	15	0	15
413507	174	12	0	12
503408	170	15	0	15
323410	365	55	11	66
303442	186	36	13	49
194620	162	49	9·7	58
634653	104	31	6·2	37
543432	41	43	2·4	45
445620	130	191	34	225
144645	47	71	7·1	78
353450	39	59	14	73
015047	62	131	9·3	140
163495	195	410	68	478
293504	64	135	9·0	144
343440	37	78	5·2	83
383438	49	103	7·3	110
403413	78	164	20	184
313439	145	517	290	807
433485	155	554	81	635
513406	44	156	11	167
684639	497	1773	127	1900
124643	134	523	35	558
643463	211	823	74	897
045607	101	517	60	577
423431	103	527	61	588
483436	83	424	173	597
473474	220	1720	309	2029
134614	250	2364	420	2784
493489	63	595	106	701

DISCUSSION

The production of only aflatoxins B_1 and B_2 by the group of *A. flavus* cultures investigated in this study was in line with the findings of other workers (Lillard, Hanlin & Lillard, 1970; Nagarajan & Bhat, 1973; Trenk & Hartman, 1970). Eighteen compounds were detected by TLC as unidentified blue or green fluorescent spots with various R_F values and were produced by 18 strains of *A. flavus*. Each strain could produce one, two, three or four fluorescent spots. The amount of aflatoxins B_1 and B_2 varied from 0 to 2784 μg/800 g substrate, levels somewhat lower than those reported previously (Hesseltine *et al.* 1966; Shotwell *et al.* 1966).

The fact that the CEX fraction was more toxic than the PEI fraction to the weanling Wistar rats was possibly due to the removal of some other toxic constitu-

Table 2. *Relationship between aflatoxin content of the substrate and its toxicity to weanling rats*

A. flavus strain no.	Calculated amount of aflatoxin administered* (in PEI; μg/rat)		Toxicity†		R_f values (and colour‡) of other products separated from PEI by TLC			
	B_1	B_2	PEI	CEX				
115602	0	0	0	+ + +				
154689	0	0	0	0				
283455	0	0	0	+	0·73(B)	0·76(B)		
075029	0·02	0	0	+ + +				
	0·05	0	0	NT				
453405	0·02	0	0	+ + +	0·42(B)	0·46(G)		
	0·05	0	0	NT				
183479	0·02	0	0	+ +	0·63(B)	0·66(G)		
333446	0·03	0	0	+ + +				
	0·05	0	0	NT				
463464	0·03	0	0	0	0·46(G)	0·58(G)	0·66(G)	
373472	0·04	0	0	+ + +				
393434	0·07	0	0	+				
363465	0·08	0	+	+ + +				
065061	0·96	0	0	+ + +	0·09(B)	0·23(B)	0·26(B)	
503408	0·96	0	0	+ + +	0·45(B)	0·59(B)		
413507	0·98	0	0	0				
634653	1·95	0·39	0	+	0·33(G)	0·46(G)		
	3·00	0·60	0	NT	0·33(G)	0·46(G)		
303442	2·27	0·82	0	+ + +				
543432	2·70	0·16	0	+ + +				
	10·50	0·60	0	NT				
194620	3·30	0·61	0	+ + +	0·73(B)			
323410	3·43	0·69	0	+ + +				
353450	3·68	0·86	0	+ + +				
	15·00	3·50	+ + +	NT				
144645	4·43	0·45	0	+ + +				
	15·00	1·50	0	NT				
343440	4·80	0·30	0	+ + +				
383438	6·10	0·60	0	+ + +				
	21·00	1·50	0	NT				
015047	8·10	0·50	0	0	0·03(B)	0·06(G)		
293504	8·90	0·60	0	+ + +				
	21·00	1·40	+ + +	NT				
513406	9·70	0·07	0	+ + +	0·53(B)	0·56(G)	0·66(G)	0·76(G)
	35·70	2·50	+	NT	0·53(B)	0·56(G)	0·66(G)	0·76(G)
403413	10·20	1·20	0	+ + +	0·43(B)	0·45(G)	0·50(G)	0·70(G)
	21·00	2·60	0	NT	0·43(B)	0·45(G)	0·50(G)	0·70(G)
445620	11·90	2·10	0	0				
163495	25·60	4·20	+	+ + +	0·16(B)	0·63(B)		
483436	26·50	10·80	0	+ + +	0·45(B)	0·59(G)	0·68(G)	
	51·00	20·80	0	NT	0·45(B)	0·59(G)	0·68(G)	
313439	32·20	18·00	+ +	+ + +				
045607	32·20	3·70	+ + +	+ + +	0·03(B)	0·06(G)		
124643	32·60	2·10	0	+ + +				
423431	32·90	3·80	+ + +	+ + +	0·03(B)	0·06(G)		
433485	34·60	5·10	+ +	+ + +				
493489	34·60	6·50	+ + +	+ + +				
643463	51·40	4·60	+ + +	+ + +	0·53(B)	0·56(G)	0·66(G)	0·76(G)
473474	107·20	19·20	+ + +	+ + +	0·42(B)	0·66(G)		
684639	110·70	7·20	+ + +	+ + +	0·45(B)	0·59(G)	0·68(G)	
	35·70	2·50	+ + +	NT	0·45(B)	0·59(G)	0·68(G)	
134614	147·70	26·20	+ + +	+ + +				

PEI = Petroleum ether-insoluble fraction CEX = Chloroform extract NT = Not tested

*The dose of the CEX or PEI fraction administered to each rat was generally equivalent to 50 g of the original rice sample, but 10 mg of the PEI fraction was given if the amount of crude toxins was inappropriate.

†Toxicity is defined by the number of rats dying from each group of five: i.e. 0—none died; +—one died; + +—two or three died; + + +—four or all five died.

‡Blue (B) or green (G) fluorescence under UV light.

Table 3. *TLC separation of products other than aflatoxins B_1, B_2, G_1 and G_2 from the petroleum ether-insoluble fraction of cultures of* Aspergillus flavus

R_f value	Fluorescence under UV light	No. of strains
0·03	Blue	3
0·06	Green	3
0·09	Blue	1
0·16	Blue	1
0·23	Blue	1
0·26	Blue	1
0·33	Green	1
0·42	Blue	2
0·43	Blue	1
0·45	Green	1
0·45	Blue	3
0·46	Green	3
0·50	Green	1
0·53	Blue	2
0·56	Green	2
0·58	Green	1
0·59	Blue	1
0·59	Green	2
0·63	Blue	2
0·66	Green	5
0·68	Green	2
0·70	Green	1
0·73	Blue	2
0·76	Green	3

In total, 18 strains gave rise to between one and four of these unidentified products, the number of strains associated with the appearance of any one spot being indicated in the final column.

ents besides aflatoxins from the CEX fraction during the defatting process. The toxicity of the PEI fraction may depend not only on the amount of aflatoxins B_1 and B_2 present but also to some extent on the presence in the extracts of certain unidentified components.

The blue fluorescent spot of R_F 0·03 combined with the green fluorescent spot of R_F 0·06 seemed to enhance toxicity, as for example in nos 045607 and 423431 compared with no. 124643 (Table 2). Where no additional compounds were detected, toxicity seemed to be dependent upon the sum of the aflatoxins B_1 and B_2 present; thus the greater toxicity of strain no. 313439 compared with no. 124643 may have been due to the higher concentration of B_2 in the former (18 μg) than in the latter (2 μg).

Where the blue and green fluorescent spots were similar, e.g. in strains no. 483436 and 684639, the fatality rate did not appear to depend entirely upon the amount of aflatoxins B_1 and B_2 The lowest level of aflatoxins that caused a high mortality with the PEI fraction was the combination of 15 μg B_1 and 4 μg B_2 (strain no. 353450), whereas the higher levels of 51 μg B_1 and 21 μg B_2 (no. 483436) caused no deaths. This might be explained by variation in the strains of *A. flavus* and the influence of other metabolites on the effects of aflatoxins B_1 and B_2. Such metabolites, if present, could not be detected by the techniques used in this study.

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Table 4. *Comparative toxicity of the chloroform extract and petroleum ether-insoluble fractions of cultures of 40 strains of* Aspergillus flavus

Fraction	Route of administration	Degree of toxicity*			
		Non-toxic	Mild	Moderate	Severe
CEX	oral	5	3	1	31
PEI	ip	25	3	2	10

*Number of strains giving rise to an extract or fraction classified, according to the number of deaths among the five rats in each group, either as non-toxic (no deaths) or as mildly (one death), moderately (two or three deaths) or severely toxic (four or five deaths).

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CITRININ MYCOTOXICOSIS IN THE RAT. I. TOXICOLOGY AND PATHOLOGY

W. H. JORDAN* and W. W. CARLTON

Department of Veterinary Microbiology and Pathology, School of Veterinary Medicine, Purdue University, West Lafayette, IN 47907

and

G. A. SANSING

Department of Microbiology, University of Texas at Austin, Austin, TX 78712, USA

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Abstract—In a series of four trials, citrinin was administered by the ip route as a single dose or in daily doses to determine its acute toxicity for the rat. The single dose LD_{50} (Trial I) for citrinin dissolved in equal parts by volume of dimethylsulphoxide and 50% ethanol was 64 mg/kg body weight. Administration of this LD_{50} dose after seven daily doses of either 32 or 48 mg citrinin/kg body weight killed only one of 19 rats (Trial II). In Trial IV, rats were killed 3, 6, 12, 24, 36 and 48 hr after receiving a single ip dose of 50 mg citrinin/kg for examination of kidneys and other tissues. The principal gross and microscopic alterations in the citrinin-treated rats were in the kidneys. Vacuolar degeneration of the renal tubular epithelium found 3 hr after treatment had progressed to extensive necrosis 33 hr later. Necrosis was the dominant lesion 36–72 hr after treatment. Regeneration of tubular epithelium, characterized by an increase in the nuclear to cytoplasmic ratio and the presence of cytoplasmic basophilia, was minimal 72 hr after treatment, but was prominent after 144 hr (Trial III).

INTRODUCTION

Citrinin is a secondary metabolite of a number of fungal species belonging to the genera Penicillium and Aspergillus (Hetherington & Raistrick, 1931; Korzybski, Kowszyk-Gindefer & Kurylowicz, 1965; Pollock, 1947; Timonin & Rouatt, 1944). The acute toxicity of citrinin for rats, guinea-pigs and rabbits (Ambrose & DeEds, 1945 & 1946), mice (Ambrose & DeEds, 1946; Jordan, Carlton & Sansing, 1977), and dogs (Carlton, Sansing, Szczech & Tuite, 1974; Kitchen, Carlton & Tuite, 1977a,b) was studied. In each of these reports the nephrotoxic effects of citrinin were demonstrated. Although the specific segments reported to be damaged by citrinin varied, principal microscopic alterations in all species were limited to the renal tubules and consisted of necrosis and desquamation of tubular epithelial cells, renal tubular dilation and the presence of protein and protein casts in the tubular lumen. The renal alterations induced by single or multiple doses of citrinin in the rat are reported below.

EXPERIMENTAL

Material. Citrinin, prepared as previously described by Carlton *et al.* (1974), was dissolved in dimethylsulphoxide and 50% ethanol (1:1, v/v; DMSO–50% EtOH) for ip administration.

Animals. Sprague–Dawley rats purchased from Harlan Industries, Cumberland, IN, were housed individually in wire-bottomed metabolism cages. Rats for Trials I–III weighed 40–60 g and those for Trial IV 190–210 g. Feed and water were supplied *ad lib.*

Collection of tissues. Rats found moribund during the experiments and survivors killed at termination were autopsied. A mid-sagittal section of the left kidney was placed in Zenker's acetic acid fixative. The right kidney, heart, lungs, and liver were collected from each rat. In addition, the spleen, brain, urinary bladder, one testicle and sections of the gastro-intestinal tract, skin and skeletal muscle were collected from selected rats. Tissues were fixed in neutral buffered 10% formalin. Fixed tissues were dehydrated, embedded in paraffin, sectioned at 4 or 6 μm and stained with Masson's trichrome (kidneys) or a haematoxylin and eosin, for histopathological examination.

Experimental design

Trial I. The single-dose LD_{50} of citrinin was determined for rats. Groups of ten rats were administered single ip doses of 50–80 mg citrinin/kg body weight. Citrinin was mixed with solvent at various concentrations so that the solvent dosage for each group was 7 ml/kg body weight. The LD_{50} and the upper and lower confidence limits (95% confidence interval) were calculated (Litchfield & Wilcoxon, 1949) on the basis of the percentages of rats dead 72 hr after treatment. Survivors were killed by ether overdose 72 hr after treatment for post-mortem examination and collection of tissues for histopathological examination.

Trial II. The effects of seven daily ip sub-lethal doses of citrinin followed by a single LD_{50} dose were studied. The daily citrinin doses were equal to either one half or three quarters of the single-dose LD_{50} determined in Trial I. Each dose regime was administered to ten weanling male rats. The LD_{50} dose was administered 24 hr after the last sub-lethal dose. Survivors were killed 72 hr after injection of the LD_{50}

* Present address: Toxicology Division, Lilly Research Laboratories, Division of Eli Lilly and Company, Greenfield, IN 46140, USA.

dose for post-mortem examination and collection of tissues for histopathological examination.

Trial III. Lesions present at day 3 were compared with those present at day 6 after a single ip dose of citrinin. Twenty weanling male rats each received a single ip injection of solvent (DMSO–50% EtOH, 5·5 ml/kg) and 28 rats received the solvent plus 50 mg citrinin/kg body weight. Ten rats from each group were killed by ether overdose on day 3 and survivors were killed on day 6 after treatment, for post-mortem examination and collection of tissues for histopathological examination. The gross and histological morphology of the kidneys from the two citrinin-treated groups and the solvent control groups were compared.

Trial IV. Early phases of the clinicopathological (see companion paper) and pathological features of citrinin-induced mycotoxicosis were studied. Each rat was given a single ip dose of the DMSO–50% EtOH solvent (5 ml/kg body weight) or of citrinin (50 mg/kg) in this solvent and placed in a wire-bottomed metabolism cage in which water and feed were supplied *ad lib*. Groups of rats were killed for collection of tissues 3, 6, 12, 24, 36 and 48 hr after treatment.

RESULTS

Trial I

Rats administered only the DMSO–50% EtOH solvent (7 ml/kg) became depressed and remained so for 0·5–1 hr. Rats administered citrinin (50–80 mg/kg) dissolved in DMSO–50% EtOH became markedly depressed and remained so for 1–2 hr. The single-dose LD_{50} determined for citrinin administered to weanling male rats by the ip route was 64 mg/kg body weight (95% confidence interval, 57–72 mg/kg).

The predominant lesions found *post mortem* involved the kidneys. Clear fluid surrounded the kidneys and was free in the abdominal cavity of rats that died 24–72 hr after treatment. The kidneys of these rats were pale and swollen, with pinpoint pale foci visible from the renal surface and numerous pale radiating streaks in the cortex visible in cross-section (Fig. 1). Renal lesions were similar in eight of 21 citrinin-treated rats killed 72 hr after treatment. The only significant extra-renal gross lesion was a dark-red discoloration of a 5- to 7-cm segment of the middle jejunum in 13 of 18 citrinin-treated rats that died within 24 hr of treatment.

Histopathologically, the pale foci seen grossly were areas of necrotic renal tubules, often involving several groups of tubules. Tubules of both the cortex and medulla were occasionally dilated and contained protein and protein (hyaline) casts, but necrotic tubules were limited to the renal cortex and the outer stripe of the outer zone of the renal medulla. Necrosis most often involved straight segments (Fig. 2) and distal convoluted tubules, although proximal convoluted tubules were also damaged in markedly affected kidneys. Regeneration of tubular epithelial cells, characterized by the presence of groups of cells with vesicular nuclei, basophilic cytoplasm and a high nuclear to cytoplasmic ratio was slight.

Protein and protein casts within renal tubules, dilation of these tubules, and necrosis and regeneration of renal tubular epithelium were more frequent in citrinin-treated rats killed 72 hr after treatment than in rats that died earlier. The lesions were also more severe in rats killed 72 hr after treatment and receiving a moderate dose (60 mg/kg) of citrinin (Table 1). Tubular necrosis contributed more to the severity of renal lesions in citrinin-treated rats than did luminal casts and luminal dilation. Renal lesions were not observed in rats that received only DMSO–50% EtOH.

Trial II

Rats developed a resistance to the toxic effects of citrinin and DMSO–EtOH solvent as a result of daily administration of the toxin and/or solvent. Behaviour following the first two to four doses of solvent and/or citrinin was similar to that observed in Trial I. Subsequent doses resulted in depression that was less marked and of shorter duration. All but one rat survived seven daily doses of citrinin at 32 or 48 mg/kg. A single LD_{50} dose of citrinin on day 8 resulted in

Table 1. *Renal lesion severity indices for rats found dead during the study or killed at termination 72 hr after ip administration of a single dose of 50–80 mg citrinin/kg (Trial I)*

Manner of death	Citrinin dose (mg/kg)	No. of rats examined	Lesion severity index* Tubular protein	Tubular dilation	Tubular necrosis	Tubular regeneration
Found dead	60	4	0·2	0·0	1·8	0·0
	70	7	0·0	0·0	1·3	0·0
	80	8	0·0	0·0	1·1	0·0
Killed 72 hr after treatment	0	15	0·0	0·0	0·0	0·0
	50	10	0·3	0·8	1·9	0·6
	60	6	0·8	1·0	3·0	0·7
	70	3	0·7	0·7	1·0	0·0
	80	2	0·5	1·0	1·5	0·5
Total found dead and killed 72 hr after treatment	0	15	0·0	0·0	0·0	0·0
	50	10	0·3	0·8	1·9	0·6
	60	10	0·6	0·6	2·5	0·4
	70	10	0·2	0·2	1·2	0·0
	80	10	0·1	0·1	1·2	0·1

*Mean of lesions, graded 0 = no change to 5 = severe.

Table 2. *Renal lesion severity indices for untreated control rats and rats given a single ip dose of citrinin (50 mg/kg) and killed 3–144 hr after treatment (Trials III and IV)*

Time after treatment (hr)	No. of rats examined	Lesion severity index*			
		Tubular protein	Tubular dilation	Tubular necrosis	Tubular regeneration
		Trial III†			
Solvent control					
72 & 144	20	0·0	0·0	0·0	0·0
Citrinin-treated					
72	10	0·6	0·6	2·3	1·0
144	15	0·5	0·6	0·3	2·3
		Trial IV‡			
Untreated control					
—	13	0·0	0·0	0·0	—
Solvent control					
3–48	76	0·0	0·0	0·0	—
Citrinin treatment					
3	11	0·1	0·0	1·8	—
6	11	0·0	0·0	2·5	—
12	13	0·6	0·2	2·6	—
24	11	1·9	0·6	2·7	—
36	15	2·2	0·7	2·7	—
48	28	2·1	1·0	2·8	—

*Mean of lesions for the given number of animals, graded from 0 = no change to 5 = severe.
†The solvent dose was 5·5 ml/kg body weight both for solvent controls and citrinin-treated animals.
‡The solvent dose was 5 ml/kg body weight both for solvent controls and citrinin-treated animals.

the death of only one rat from the 32-mg/kg group and no rats from the 48-mg/kg group.

Renal lesions were similar to those described in Trial I. Tubular necrosis contributed most to the severity of lesions observed in the group primed with daily doses of 32 mg/kg while regeneration of tubular epithelium was the most prominent change observed in the group primed with 48 mg citrinin/kg before receiving the higher dose of 64 mg citrinin/kg.

Trial III

The clinical and pathological features of the toxicosis induced by a single ip dose of 50 mg citrinin/kg were like those observed in Trial I. Renal lesions were observed in nine of the ten citrinin-treated rats killed on day 3 and in 12 of the 15 rats killed on day 6. Differences were found between the two citrinin-treated groups in the types of renal lesions, as necrosis of the tubular epithelium was the dominant lesion in the kidneys of rats killed on day 3, whereas regeneration of tubular epithelium was the dominant change in the kidneys of rats killed on day 6 (Table 2).

Trial IV

As observed in Trial I, citrinin-treated rats became markedly depressed and remained so for 1–2 hr and solvent control rats were depressed for 30 min to 1 hr after treatment.

Gross lesions were observed neither in untreated and solvent-control rats killed during the 48-hr study nor in citrinin-treated rats killed 3 hr after treatment. Mild to moderate ascites was found in 80% of the rats killed 6–48 hr after treatment. Clear fluid (2–10 ml) was present in the abdominal cavity and fluid surrounded the kidneys of these rats. Kidneys of rats with ascites were pale and swollen. Pinpoint pale foci visible from the renal cortical surface were connected to narrow pale radiating streaks in cross-sections of the renal cortex. Such lesions were observed in 25% of the rats killed after 24 hr and in 50% of those killed 36 and 48 hr after treatment.

Histopathological alterations were observed only in citrinin-treated rats and included degeneration and necrosis of cortical tubular epithelium, the presence of protein and protein casts in tubular lumens and renal tubular dilation. The lesion severity index (Table 2) was based on the relative number of renal tubules affected, the proportion of rats affected, the percentage of cells affected, the amount of protein and the degree of dilation of those tubules. The mean grade of tubular necrosis, on the basis of 0 = no change to 5 = severe (complete destruction of cortical tubules), was 1·8 by 3 hr and 2·5 by 6 hr after treatment, a plateau at 2·5–2·8 being maintained from the latter time until the final killing at 48 hr. Renal tubular dilation and the presence of protein and protein casts in tubules were rarely observed 3 and 6 hr after treatment. Tubular dilation was observed in two and tubular protein in eight of the 13 rats examined 12 hr after treatment and in the majority of the rats at 24–28 hr after treatment. The mean gradings for the severity of tubular dilation and tubular protein 24–48 hr after treatment were 0·6–1·0 and 1·9–2·1 respectively (Table 2).

The earliest histopathological alteration of renal tubular epithelial cells was cytoplasmic vacuolation. This was followed in approximate chronological order, by nuclear pyknosis, condensation of cytoplasm, karyorrhexis, karyolysis and dissolution of cytoplasm. Prominent, irregularly shaped vacuoles (Fig. 3) were observed in the renal tubular epithelial cells in 10 of 11 rats killed 3 hr after treatment. A few pyknotic nuclei were observed at this time and condensation

of the cytoplasm of epithelial cells was observed in an occasional tubule of two rats killed 3 hr after treatment. The degree of vacuolar change was greater by 6 hr after treatment and, at 12 hr, some vacuoles were partially filled with finely granular eosinophilic material. Nuclear pyknosis was evident in vacuolated epithelial cells 24 hr after treatment (Fig. 4). Vacuolar change was not prominent in tubular epithelium at 36 and 48 hr after treatment, but necrosis became dominant and affected indivudual cells and groups of tubular epithelial cells 36–72 hr after treatment. Necrotic cells were round with small dense hyperchromatic (pyknotic) nuclei and intensely red (trichrome stain) cytoplasm and were not in contact with adjacent normal epithelial cells. Necrosis of groups of tubular epithelial cells was characterized by nuclear pyknosis, karyorrhexis or karyolysis and by loss of brush border and dissolution of cytoplasm (Fig. 5). Degeneration and necrosis of epithelium affected tubules of the cortex cortices, labyrinthine cortex and the outer stripe of the outer zone of the medulla. Based on this distribution and other anatomical features such as depth of brush border and height of epithelial cells (Rhodin, 1974; Rouiller, 1969), affected tubules were identified as distal convoluted tubules and straight segments of proximal and/or distal tubules.

Extra-renal lesions associated with citrinin administration included increased necrosis of individual lymphoid cells in the spleen and thymus and increased mitotic activity in hepatocytes of citrinin-treated rats.

DISCUSSION

Citrinin given in one to seven doses was nephrotoxic to rats as revealed by gross and histopathological alterations. Despite differences in experimental procedures, comparison of our data with that reported by other investigators (Ambrose & DeEds, 1946; Carlton *et al.* 1974; Kitchen *et al.* 1977a,b; Thacker, Carlton & Sansing, 1977) suggests that the rat was less susceptible to the toxic effects of citrinin than the guinea-pig, beagle dog or rabbit, but was more susceptible than the mouse (Jordan *et al.* 1977).

Rats became less susceptible to the toxic effects of citrinin when given seven daily mildly toxic doses of citrinin. This was demonstrated by the low mortality when this group was given a dose equivalent to the LD_{50} on day 8 of the study. Such a response to citrinin administration may be a species response rather than one determined by the mycotoxin, since the response to multiple doses of citrinin has varied with the species tested. Thus, decreasing susceptibility was observed in guinea-pigs given daily doses of citrinin (Thacker *et al.* 1977), but multiple doses were additive in toxicity when administered to the hamster. The mechanism responsible for the induction of resistance to a lethal dose by multiple sublethal doses of citrinin was not investigated in these studies, but may be related to re-population of tubules by resistant cells or by the induction of enzyme systems more efficient in citrinin detoxification (Orrenius, 1965).

The earliest observed histopathological alteration in the kidneys was vacuolation of tubular epithelial cells, which was detected much more readily in Zenker's-fixed than in formalin-fixed tissues. Vacuolar change 6–24 hr after treatment was accompanied by loss of cytoplasmic eosinophilia and by nuclear pyknosis indicative of necrosis. Such changes have also been described in the kidneys of dogs (Carlton *et al.* 1974; Kitchen *et al.* 1977b) and guinea-pigs (Thacker *et al.* 1978) treated with multiple doses of citrinin. These structural changes are non-specific and have been described in kidneys damaged by a variety of noxious agents. Vacuolation, the most prominent morphological alteration observed 3–24 hr after treatment in citrinin-treated rats, has been described in association with hypokalaemia (Sarkar & Levine, 1975). However, hypokalaemia was apparently not responsible for vacuolation in rats poisoned with citrinin, as serum concentrations of potassium were not reduced but tended to be slightly above those of control rats (Jordan, Carlton & Sansing, 1978). The contents of the vacuoles in the renal tubular cells of our rats were not determined precisely as special stains were negative for glycogen and lipid. However, accumulation of fluid in the intracellular compartments of renal tubular cells due to altered function of the sodium pump (Trump & Arstila, 1975) may have been responsible.

The change from epithelial vacuolation and nuclear pyknosis at 24 hr after treatment to dissolution of cytoplasm and loss of nuclei after 36 hr was accompanied by a dramatic increase in activities of LDH and GOT in the urine (Jordan *et al.* 1978), no doubt arising from the damaged renal tubular cells. Activities of urinary enzymes declined 42 and 48 hr after treatment, coincident with stabilization of cellular breakdown.

The histological morphology of the kidneys of citrinin-treated rats 72 hr after treatment was very similar to that after 48 hr except for very slight evidence of regeneration of tubular epithelium at 72 hr. The paucity of necrotic tubular epithelium and the abundance of regenerating epithelial cells 144 hr after treatment compared to the extensive lesions observed earlier clearly demonstrated the remarkable ability of renal tubular epithelium to recover after a severe insult. Rapid regeneration of the renal tubular cells may be responsible, at least in part, for the apparent resistance of the rat and mouse (Jordan *et al.* 1977) to multiple doses of citrinin.

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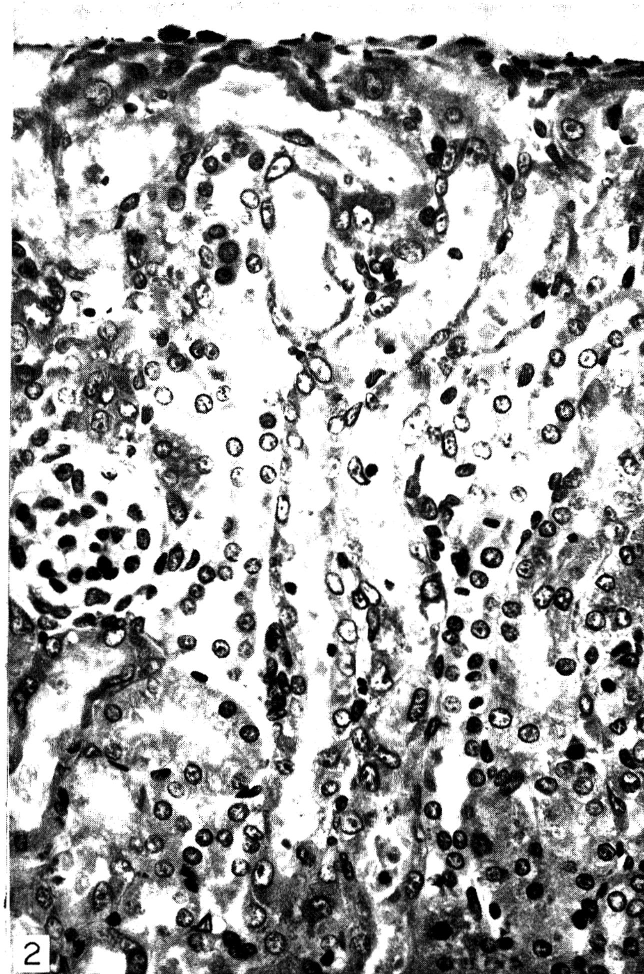

Fig. 1. Cross-section of the renal cortex of a kidney from a rat that died after citrinin treatment, showing pale radiating streaks.

Fig. 2. Necrosis of tubules of cortex cortices and straight segments of tubules of labyrinthine cortex in a rat given a single ip dose of 50 mg citrinin/kg. Haematoxylin and eosin ×250.

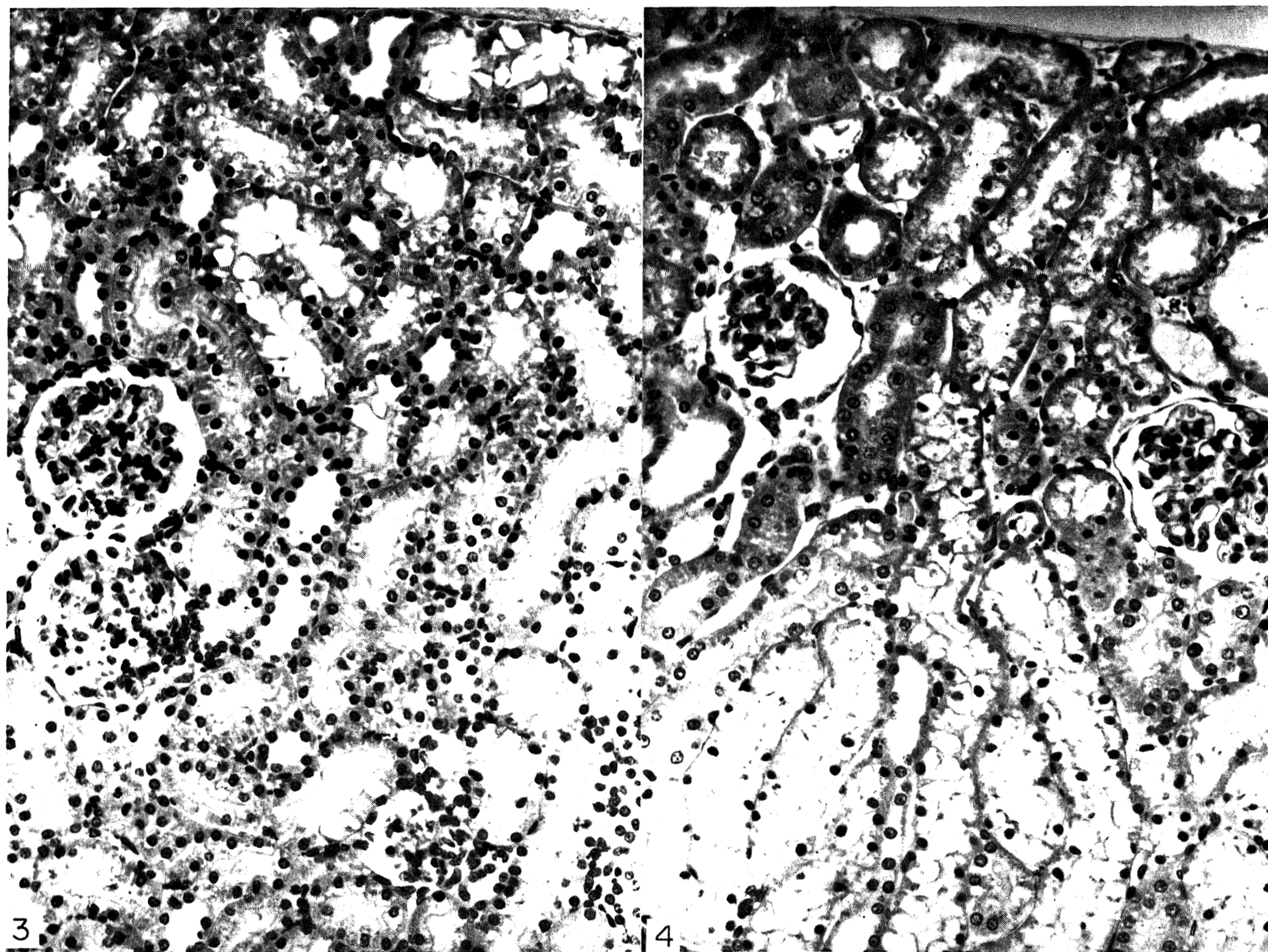

Fig. 3. Cytoplasmic vacuolation in several groups of renal cortical tubular epithelial cells of a rat 3 hr after a single ip dose of 50 mg citrinin/kg (Trial IV). Haematoxylin and eosin × 160.

Fig. 4. Cytoplasmic vacuolation and nuclear pyknosis of renal tubular epithelium of a rat killed 24 hr after a single ip dose of 50 mg citrinin/kg (Trial IV). Haematoxylin and eosin × 160.

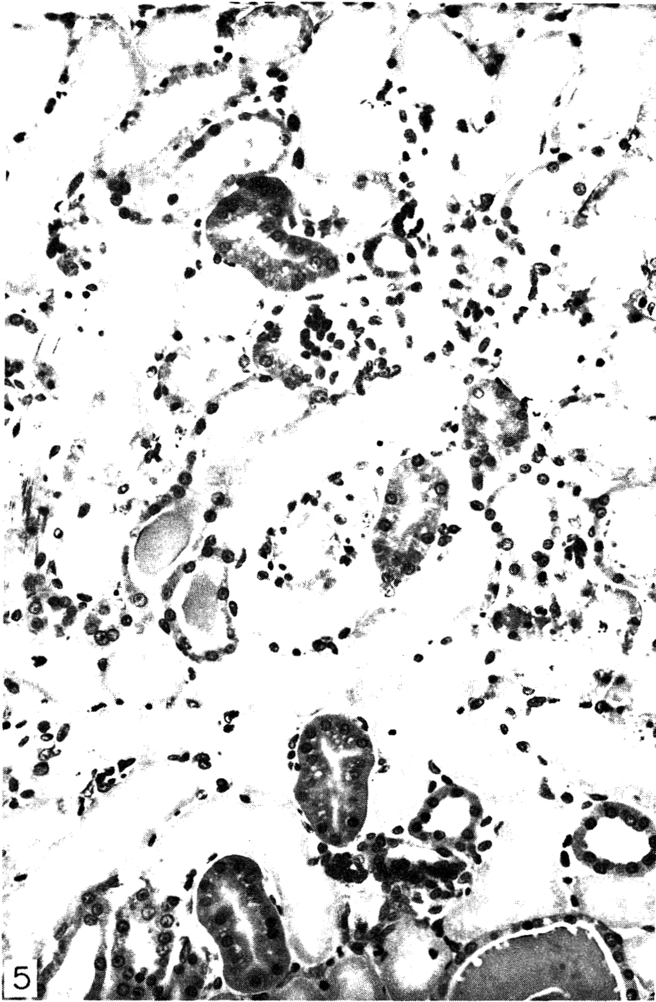

Fig. 5. Marked renal tubular necrosis and hyaline casts in a rat killed 36 hr after a single ip dose of 50 mg citrinin/kg (Trial IV). Haematoxylin and eosin × 160.

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CITRININ MYCOTOXICOSIS IN THE RAT. II. CLINICOPATHOLOGICAL OBSERVATIONS

W. H. JORDAN* and W. W. CARLTON

Department of Veterinary Microbiology and Pathology, School of Veterinary Medicine, Purdue University, West Lafayette, IN 47907,

and

G. A. SANSING

Department of Microbiology, University of Texas at Austin, Austin, TX 78712, USA

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Abstract—Sprague-Dawley rats weighing about 200 g were given either a single ip dose of 50 mg citrinin/kg or the dimethylsulphoxide-50% ethanol vehicle alone in a volume of 5 ml/kg and several clinicopathological parameters were examined. Alterations found during the first 48 hr after administration of citrinin were indicative of nephrosis. Concentrations of glucose and blood were increased in the urine of citrinin-treated rats and the concentrations were greatest 3 hr after treatment. Protein in the urine increased from a mean of less than 2 + in untreated rats to 3 + 48 hr after citrinin treatment (solvent control = 1 +). A decrease in protein concentration in the urine of solvent-control rats was due to diuresis induced by the dimethylsulphoxide-ethanol solvent. The mean hourly excretions of chloride and sodium in the urine of citrinin-treated rats were 1·5-2·0 times those of solvent-control rats, while potassium excretion was only slightly greater (101 compared with 92 mequiv/litre) in the citrinin-treated than in the solvent-control rats. Activities of glutamic-oxalacetic transaminase and lactic dehydrogenase in the urine of citrinin-treated rats were 15-20 times those of solvent control rats 36 hr after treatment. The blood urea nitrogen increased to 114 mg/100 ml 48 hr after treatment, about five times the normal concentration. Activities of lactic dehydrogenase and glutamic-oxalacetic transaminase in the serum were only slightly elevated in citrinin-treated rats. Serum potassium concentration was elevated and serum sodium and chloride concentrations were reduced.

INTRODUCTION

Citrinin, reviewed by Scott (1977), is a nephrotoxic secondary metabolite of a number of fungal species belonging to the genera Penicillium and Aspergillus. Clinicopathological features of citrinin-induced mycotoxicosis in the beagle dog (Carlton, Sansing, Szczech & Tuite, 1974; Kitchen, Carlton & Tuite, 1977) and in the pig (Friis, Hasselager & Krogh, 1969) were indicative of nephrosis.

The toxicological and histopathological features of citrinin toxicosis in the rat are reported in a companion paper (Jordan, Carlton & Sansing, 1978). This paper reports the clinicopathological data obtained following administration of citrinin to rats.

EXPERIMENTAL

Material. Citrinin, prepared as previously described by Carlton *et al.* (1974), was dissolved in dimethylsulphoxide-50% ethanol (1:1, v/v; DMSO-50% EtOH) for administration by the ip route.

Animals. Sprague-Dawley rats weighing 190-210 g were purchased from Harlan Industries, Cumberland, IN, and were housed individually in wire-bottomed metabolism cages. Feed and water were supplied *ad lib.*

Design of experiment. Early phases of the pathological clinicopathological features of citrinin-induced mycotoxicosis were studied. Test rats were given a single ip dose of 50 mg citrinin/kg in a mixture of DMSO-50% EtOH as solvent (5 ml/kg body weight). Control rats were given only the solvent. Both groups were placed in wire-bottomed metabolism cages in which water and feed were supplied *ad lib.* Urine from selected rats was collected 3 and 6 hr after treatment and at 6-hr intervals until the rats were killed at or before 48 hr. Groups of at least ten rats were killed for collection of blood and tissues 3, 6, 12, 24, 36 and 48 hr after treatment. In addition, water intake and urine output were recorded for 14 untreated control, 10 solvent-control and 12 citrinin-treated rats, which were also killed 48 hr after treatment.

Urine was collected over periods of 3 or 6 hr at the above intervals. Colour and clarity were observed. Specific gravity was determined with a refractometer. The pH of the urine was recorded and testing for the presence of protein, glucose, ketones, bilirubin, urobilinogen and blood was carried out using diagnostic reagent strips (Multistix, Ames Co., Elkhart, IN). Unstained urinary sediment was examined by light microscopy after centrifugation. Sodium and potassium concentrations were determined by flame photometry and urinary chloride levels were measured with a chloride meter (Cotlove, Trantham & Bowman, 1958). The activities of lactic dehydrogenase (LDH) and glutamic-oxalacetic transaminase

* Present address: Toxicology Division, Lilly Research Laboratories, Division of Eli Lilly and Company, Greenfield, IN 46140, USA.

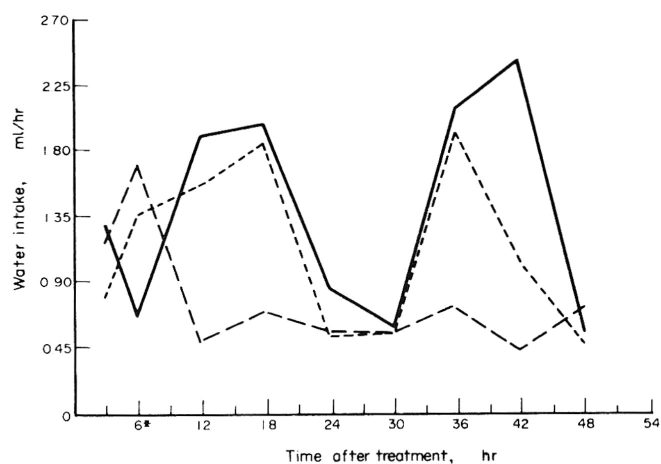

Fig. 1. Water intake of untreated control rats (——), solvent-control rats (-----) and rats given a single ip dose of 50 mg citrinin/kg (— — —).

(GOT) in the urine were determined with commercial kits (Boehringer Mannheim Corporation, New York). The change in light absorbance for both enzyme reactions was recorded over a 3-min period at 336 nm and 30°C (Beckman Model 25, Kinetic System, Fullerton, CA).

Blood was collected from the right ventricle after the thorax had been opened under ether anaesthesia, some being placed in tubes containing potassium EDTA as anticoagulant and some in other tubes without anticoagulant to allow clot formation. After centrifugation, serum was sampled for chemical, electrolyte and enzyme determinations. Blood urea nitrogen (BUN) levels were determined by a paper-chromatographic technique (Urograph-Warner Chilcott Laboratories, Morris Plains, NJ). Concentrations of sodium, potassium and chloride and activities of LDH and GOT in the serum were determined in the same manner as for the urine. Total erythrocyte and leucocyte counts were made with an electronic counting device (Coulter Counter, Model Fn-Coulter Electronics Inc., Hialeah, FLA). Haemoglobin concentrations were determined by a cyanmethaemoglobin method (Coulter Haemoglobinometer, Coulter Electronics, Inc., Hialeah, FLA), packed cell volumes (PCV) by a standard microhaematocrit method and total protein with a refractometer.

Statistical analysis of clinicopathological data was completed (using the programme *Statistical Package for the Social Sciences*, 2nd Ed.) at the Purdue University computer centre. Differences between observed means were considered statistically significant when $P \leqslant 0.01$.

RESULTS

Citrinin-treated rats became markedly depressed and remained so for 1–2 hr and solvent-control rats were depressed for 0·5–1 hr after treatment. Diurnal variations in water intake were observed in untreated and solvent-control rats, while water intake in citrinin-treated rats was relatively constant and low (Fig. 1). Only minor variations were observed in the urine output of untreated rats, but both solvent-control and citrinin-treated rats produced large quantities of urine 6–18 hr after treatment (Fig. 2). Although it

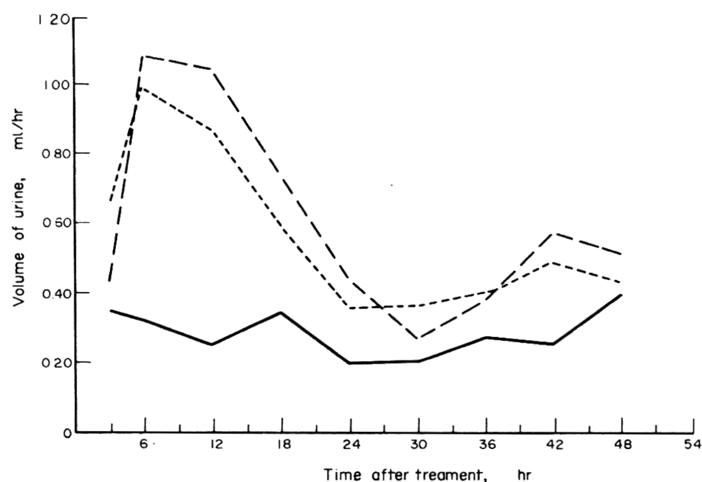

Fig. 2. Urine output of untreated control rats (——), solvent-control rats (-----) and rats given a single ip dose of 50 mg citrinin/kg (— — —).

was not monitored closely, food intake of citrinin-treated rats appeared to be lower than that of control rats.

The urine of test rats was very light yellow to moderately yellow whereas the urine of control rats was generally darker yellow. The urine from both groups was generally clear and the slight turbidity occasionally found in samples from control rats was due to the presence of numerous crystals. After 18 hr, the urine of citrinin-treated rats was sometimes turbid owing to the presence of epithelial cells, cellular debris and occasional smooth or granular casts.

The amounts of urine available were small and thus little effort was made to quantify the urinary sediment. Epithelial cells and erythrocytes were present in the urine of citrinin-treated rats as early as 3 hr after treatment. Increased numbers of epithelial cells and occasional granular and hyaline casts and decreased numbers of erythrocytes were observed in samples of urine obtained between 6 and 48 hr.

Urinary specific gravity was decreased slightly 3 hr after treatment of rats with citrinin and 6 hr after treatment in solvent-control rats. The mean urinary specific gravity of solvent-control rats gradually increased to a value similar to that of untreated control rats, but it remained low in the citrinin-treated group. Ketones, bilirubin and urobilinogen were not detected in either control or citrinin-treated rats.

Urinary pH was decreased in rats given the DMSO–50% EtOH solvent alone or in combination with citrinin. The reduction was about one pH unit 3 hr after treatment in solvent-control rats, but 48 hr after treatment, the pH of the urine of solvent-control rats was similar to that of untreated control rats (7·3). The pH of the urine of citrinin-treated rats was 0·7–1·2 units below that of solvent-control rats 18–48 hr after treatment, but these differences were statistically significant ($P < 0{\cdot}01$) only at 18 and 48 hr.

Urinary protein concentrations (Table 1) in citrinin-treated rats increased from a mean of slightly less than 2 + (based on a scale of 0 to 4 +) to a mean somewhat less than 3 +, while those of solvent-control rats increased from trace amounts 3 hr after treatment to slightly over 1 + at 48 hr. The urinary protein concentration of untreated control rats was slightly less than 2 + (solvent-induced diuresis and dilution of protein in urine).

The concentration of glucose in the urine of citrinin-treated rats had increased by 3 hr after treatment, but the increase was not so great at 6–48 hr as at 3 hr. It was, however, greater at all times in treated than in untreated and solvent-control rats; the urine samples from rats of these latter groups rarely contained detectable glucose (Table 1).

Blood was present in the urine of some of the untreated control, solvent-control and citrinin-treated rats at each collection, but was present in greater concentrations and at a greater frequency in citrinin-treated than in control rats (Table 1). This difference was statistically significant only for the 3-hr collection.

The concentrations of sodium, potassium, and chloride in the urine of rats were variable within groups as well as between principals and controls (Table 2). The concentrations of all three electrolytes were significantly ($P \leqslant 0{\cdot}01$) lower in citrinin-treated than in control rats 36–48 hr after treatment.

Activities of the enzymes GOT and LDH in the urine were increased greatly in rats treated with citrinin as compared to solvent-dosed controls. Enzymic activity peaked 36 hr after treatment, but remained high at the 48-hr terminal collection. A

Table 1. *Concentrations of protein, glucose, and blood in urine collected at various times after treatment from control and citrinin-treated rats*

Experimental group	Time after treatment (hr)	Total no. of samples	No. of urine samples with a protein concentration index of:						No. of urine samples with a glucose concentration index of:				No. of urine samples with a blood concentration index of:			
			0	tr	1+	2+	3+	4+	0	1+	2+	3+	0	1+	2+	3+
Untreated control	0	29	9	0	8	12	0	0	27	2	0	0	21	7	1	0
Solvent control	3	30	0	6	23	1	0	0	30	0	0	0	25	4	0	1
(DMSO–	6	43	10	15	18	0	0	0	43	0	0	0	38	2	2	1
50% EtOH,	12	31	9	8	14	0	0	0	31	0	0	0	26	4	1	0
5 ml/kg)	18	28	5	1	18	2	2	0	27	1	0	0	22	1	2	3
	24	30	7	5	15	2	1	0	30	0	0	0	27	1	1	1
	30	15	3	1	10	1	0	0	15	0	0	0	9	2	2	2
	36	21	0	0	11	10	0	0	21	0	0	0	16	2	3	0
	42	12	0	1	10	1	0	0	12	0	0	0	10	1	1	0
	48	20	1	1	16	1	1	0	20	0	0	0	18	1	1	0
Citrinin-treated	3	36	1	1	18	14	2	0	4	0	3	29	3	18	7	8
(50 mg/kg)	6	62	0	6	20	29	7	0	9	7	9	37	10	35	14	3
	12	41	1	0	14	16	9	1	21	11	3	6	14	19	5	3
	18	35	0	1	8	9	17	0	26	3	2	4	19	15	1	0
	24	36	1	1	5	6	22	1	23	7	3	3	17	17	2	0
	30	20	1	0	3	2	14	0	14	3	1	2	7	12	0	1
	36	32	1	0	7	2	22	0	19	4	3	6	12	18	0	2
	42	22	0	0	3	3	15	1	14	3	1	4	6	11	5	0
	48	29	0	1	3	2	21	2	20	3	1	5	10	13	6	0

tr = trace

Table 2. *Concentrations of electrolytes in urine collected at various times after treatment from untreated and solvent control, and citrinin-treated rats*

Experimental group	No. of animals tested	Time after treatment (hr)	Sodium concentration (mequiv/litre)	Potassium concentration (mequiv/litre)	Chloride concentration (mequiv/litre)
Untreated control	10	0	188·2 ± 31·5	267·8 ± 63·0	210·9 ± 34·9
Solvent control	30	3	23·4 ± 20·4	91·4 ± 37·9	24·9 ± 20·0
(DMSO–50% EtOH,	28	6	9·6 ± 14·5	99·6 ± 33·1	18·6 ± 16·2
5 ml/kg)	22	12	43·1 ± 52·8	158·6 ± 55·7	60·8 ± 44·4
	20	18	67·6 ± 56·6	185·2 ± 49·8	106·7 ± 59·8
	22	24	88·0 ± 65·0	176·1 ± 67·8	125·7 ± 72·5
	15	30	131·3 ± 83·2	188·8 ± 92·8	140·9 ± 78·7
	22	36	92·3 ± 58·1	179·8 ± 53·0	130·7 ± 67·5
	12	42	132·9 ± 62·9	243·7 ± 70·3	133·3 ± 43·1
	17	48	137·7 ± 67·9	163·5 ± 60·2	133·1 ± 67·1
Citrinin-treated	38	3	35·2 ± 27·6	90·1 ± 42·1	29·4 ± 34·8
(50 mg/kg)	43	6	33·7 ± 20·3	71·6 ± 26·5	16·1 ± 14·1
	38	12	31·1 ± 25·7	61·3 ± 28·7	20·4 ± 20·4
	34	18	34·7 ± 29·2	74·8 ± 34·1	29·2 ± 33·0
	29	24	28·4 ± 20·4	90·4 ± 45·3	25·4 ± 26·5
	18	30	30·5 ± 33·8	98·1 ± 41·6	30·9 ± 37·4
	31	36	20·6 ± 21·0	91·7 ± 50·2	22·9 ± 25·1
	23	42	21·6 ± 29·6	102·1 ± 54·6	20·8 ± 22·8
	26	48	17·0 ± 16·7	95·5 ± 48·4	21·0 ± 18·9

small spike of increased activities of these enzymes was noted 3 hr after treatment (Fig. 3).

The mean hourly urinary excretion of potassium, chloride and sodium was reduced and the urinary excretion of enzymes (GOT and LDH) was markedly higher in citrinin-treated than in solvent-control rats. The values were based on calculations using urine volumes and electrolyte and enzymic concentrations in urine samples collected at nine intervals over a 48-hr period from five citrinin-treated and five solvent-control rats (Table 3).

Activities of these enzymes were also increased in the serum of citrinin-treated rats, but the elevations in the serum were not as great as those found in the urine. LDH activities in serum peaked 3 and 36 hr after treatment (Fig. 4). LDH activities were also increased in solvent-control rats at these same collection periods, but were much below those of the citrinin-treated rats. Differences in LDH activities in serum between citrinin-treated and solvent-control rats were statistically significant 3, 36 and 48 hr after treatment. A small increase in serum GOT activity was also noted in solvent-control rats 3 hr after treatment, but the level had returned nearly to pretreatment values by the 48-hr determination. A statistically significant increase in serum GOT activity was found in citrinin-treated rats 3 hr after treatment and the increased activity persisted throughout the 48-hr trial period (Fig. 5).

In citrinin-treated rats, BUN increased throughout the study to a mean of 114 mg/100 ml at 48 hr. The BUN of solvent-control rats remained relatively constant at 13–24 mg/100 ml.

Serum electrolyte concentrations varied somewhat

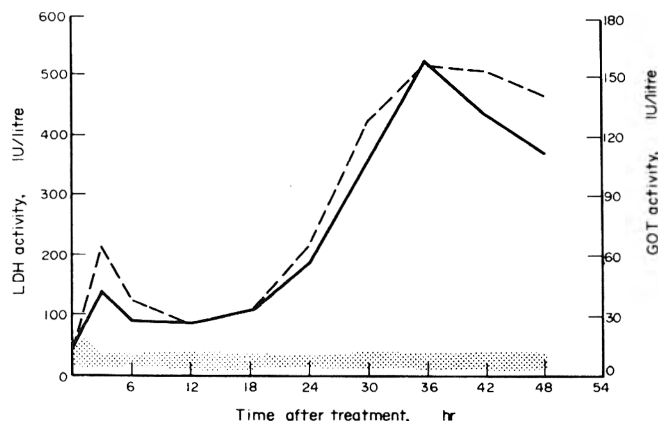

Fig. 3. Mean activities of lactic dehydrogenase (LDH; ---) and glutamic–oxalacetic transaminase (GOT; ——) in urine from rats given a single ip dose of 50 mg citrinin/kg. The shaded portion represents the 99% confidence interval of the means of the LDH and GOT activities of the solvent-control rats, including the extremes of both enzymes.

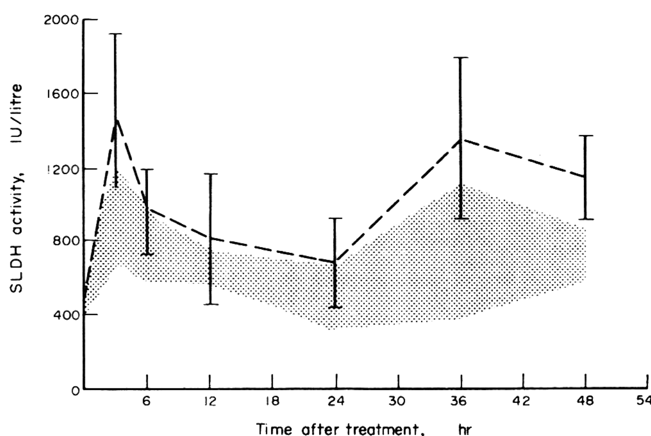

Fig. 4. Mean activities and 99% confidence intervals of serum lactic dehydrogenase (SLDH) in rats given a single ip dose of 50 mg citrinin/kg. The shaded area represents the 99% confidence interval of the mean SLDH activities of solvent-control rats.

Table 3. *Mean hourly excretion of electrolytes and enzymes in urine from five solvent-control and five citrinin-treated rats collected at nine intervals over a 48-hour period*

Urinary electrolyte or enzyme	Solvent-control rats	Citrinin-treated rats
Sodium (mequiv/hr)	54·2	22·9
Potassium (mequiv/hr)	100·6	91·7
Chloride (mequiv/hr)	56·6	35·7
UGOT (mU/hr)	6·7	51·6
ULDH (mU/hr)	15·9	214·3

UGOT = Urinary glutamic–oxalacetic transaminase
ULDH = Urinary lactic dehydrogenase

(Table 4) and most differences between citrinin-treated and solvent-control rats were not statistically significant. However, serum concentrations of sodium and chloride were generally slightly lower in treated than in control rats, and serum potassium concentrations were generally higher. The differences between treated and control values for serum potassium were statistically significant only at 24 hr after treatment.

Total serum-protein was markedly reduced (from 7·4 to 5·6 g/100 ml) 3 hr after treatment in both solvent-control and citrinin-treated rats. Mean serum-protein values gradually increased at the other observation periods and were near pretreatment values at the 48-hr terminal collection.

Mean erythrocyte counts and PCV readings of solvent-control rats were reduced 3 hr after treatment and those of citrinin-treated rats were slightly increased, but they were not significantly different from pretreatment values. Erythrocyte counts and PCV values of solvent-control and citrinin-treated rats were not significantly different from pre-treatment values 6 hr after treatment. Haemoglobin values generally paralleled those for erythrocytes and PCV. The only statistically significant difference between treated rats and controls was in the haemoglobin concentration after 3 hr. Leucocyte counts of citrinin-treated rats tended to be slightly lower than those of control rats, but these differences were not statistically significant.

DISCUSSION

A variety of clinicopathological parameters were

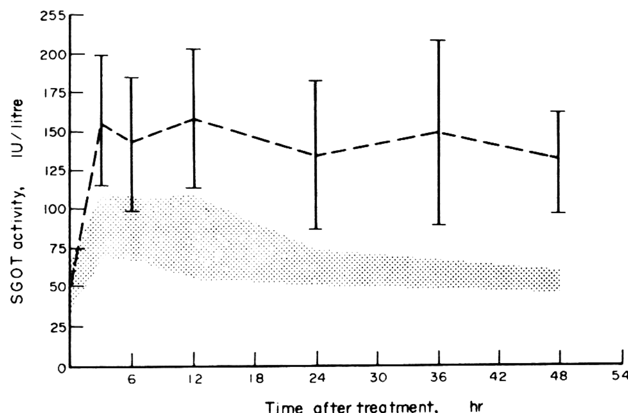

Fig. 5. Mean activities and 99% confidence intervals of serum glutamic–oxalacetic transaminase (SGOT) in rats given a single ip dose of 50 mg citrinin/kg. The shaded area represents the 99% confidence interval of the mean SGOT activities of solvent-control rats.

Table 4. *Concentrations of electrolytes in serum of untreated, solvent control and citrinin-treated rats killed at various times after treatment*

Treatment	No. of animals tested	Time after treatment (hr)	Sodium concentration† (mequiv/litre)	Potassium concentration† (mequiv/litre)	Chloride concentration† (mequiv/litre)
Untreated control	10	0	148·2 ± 2·1	5.9 ± 0.7	95·9 ± 6·4
Solvent control	10	3	139·6 ± 3·6	5·1 ± 0·6	96·0 ± 1·1
(DMSO–50% EtOH.	10	6	144·4 ± 1·7	5·2 ± 0·6	100·2 ± 2·5
5 ml/kg)	12	12	144·6 ± 1·8	6·1 ± 0·9	97·3 ± 4·2
	10	24	146·7 ± 2·6	5·8 ± 0·8	100·6 ± 9·3
	11	36	143·2 ± 3·4	5·4 ± 0·5	96·1 ± 4·2
	22	48	144·1 ± 5·3	5·7 ± 0·5	95·7 ± 5·1
Citrinin-treated	11	3	136·3 ± 5·2	5·3 ± 0·5	92·5 ± 3·5
(50 mg/kg)	11	6	139·1 ± 4·5	5·7 ± 0·8	96·5 ± 4·1
	13	12	139·8 ± 5·5	7·0 ± 1·8	96·2 ± 4·0
	10	24	141·1 ± 6·4	7·8* ± 3.0	97·1 ± 4·3
	15	36	140·6 ± 5·1	6·8 ± 1·9	97·1 ± 6·1
	28	48	135·2 ± 11·5	7·1 ± 2·1	91·5 ± 12·6

† Values are means ± standard deviation for the number of animals shown.
The value marked with an asterisk differs significantly from the control (*$P < 0·01$).

altered in rats as early as 3 hr after a single dose of citrinin and these alterations persisted to a varying extent throughout the 48-hr study period. The most prominent clinicopathological changes included a rise in BUN concentration, increased activities of GOT and LDH in urine and serum, increased concentrations of urinary glucose and protein and decreases in concentration and total excretion of urinary electrolytes. All alterations were indicative of renal damage. No haematological changes were observed

Most of the enzyme activity in the urine originated from damaged tubular epithelial cells, although some activity may have been contributed by erythrocytes. The source of the blood was not established, but small quantities were observed in the urine of untreated and solvent-control as well as of citrinin-treated rats throughout the experiment. Enzymes released into the serum did not affect urinary enzyme activities because of their inability to cross glomerular membranes owing to their high molecular weight (Amador & Wacker, 1970).

The source of the slight increase in activity of LDH and of the moderate increase in activity of GOT in the serum was not apparent. Increased activity of LDH, but not GOT, was detected in the serum of dogs given daily doses of 10 mg citrinin/kg body weight (Kitchen *et al.* 1977) but no increase in activity of either GOT or LDH was observed in the serum of dogs given daily doses of 20 or 40 mg citrinin/kg body weight (Carlton *et al.* 1974). These differences in results may be due to species differences in susceptibility to citrinin, to differences in the solvent used or to differences in citrinin dosage, or they may result from differences in the time between treatment and sample collection. Increased activities of LDH and GOT most often arise from damage to the liver and muscle (Coles, 1974; Henley, Schmidt & Schmidt, 1966), but no gross or microscopic evidence of degeneration was observed in these or other extra-renal tissues of our rats to explain the increased activities of these enzymes in the serum. It has been demonstrated that enzymes may be released from cells that appear morphologically intact (Henley *et al.* 1966; Zierler, 1958) and such "leaky" cells could have been the origin of the increased activities. It is possible that necrosis of tubular epithelium was accompanied by sufficient damage to tubular basement membranes of rats to allow leakage of enzymes into the interstitium, to be picked up by the blood stream. This enzymic activity may have been augmented by inapparent damage to the heart or liver of citrinin-treated rats. Determinations of isoenzyme patterns could have been of value in determining the source of the increased activities of LDH in the serum of citrinin-treated rats. (Cornish, Barth & Dodson, 1970), but these determinations were not made.

Functional changes support the histological observation that necrosis involved cells of the distal segment of the nephron. This segment, under normal conditions, concentrates urine by the reabsorption of solute-free water, secretes potassium and hydrogen ions and reabsorbs small quantities of sodium, which escape reabsorption in the proximal portions of the nephron (Coles, 1974; Guyton, 1976). Damage to this region would result in a decreased ability to concentrate urine, decrease in acidification of urine, impaired ability to maintain sodium balance with restricted dietary intake or excessive extrarenal losses (Kashgarian, Hayslet & Spargo, 1977) and retention of potassium (Coles, 1974). In both the solvent-control and citrinin-treated rats, the specific gravity of the urine was reduced and the urine was more acid, these changes being more marked and persistent in the citrinin-treated rats. The mechanism responsible for the increased acidity of the urine was not examined. This change may have been caused partly by the presence of acidic citrinin in the urine, although the low concentrations of citrinin (highest 42 μg/ml) reported in the urine of the dog (Damodaran, Ramadoss & Shanmugasundaram, 1973; Wang & Ting, 1950) would account for only a minor increase in urinary acidity. Damage to distal convoluted tubular epithelial cells may also have contributed to urinary acidity owing to decreased reabsorption of hydrogen ions (Coles, 1974). Decreased excretion of sodium and chloride in the urine may have been caused by de-

creased dietary intake, but the slightly reduced concentrations of sodium and chloride and the increased concentration of potassium in the serum were probably the result of failure of the normal active exchange of sodium for potassium in the damaged distal tubules (Coles, 1974).

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MYCOTOXICOSIS PRODUCED IN RATS BY CULTURAL PRODUCTS OF AN ISOLATE OF *ASPERGILLUS OCHRACEUS*

J. L. ZIMMERMANN and W. W. CARLTON
Department of Veterinary Microbiology, Pathology and Public Health, School of Veterinary Medicine,
and
J. TUITE
Department of Botany and Plant Pathology, School of Agriculture, Purdue University, West Lafayette, IN 47907, USA

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Abstract—The toxicity of an isolate of *Aspergillus ochraceus* was examined in weanling male Sprague-Dawley rats fed diets containing a rice culture or fungal mat of the organism for 5 wk. The ground rice culture was mixed with a commercial purified diet at concentrations of 5, 7·5 and 10% and the fungal mat at concentrations of 1, 2 and 3%. In groups fed the rice-culture diets, weight gains were reduced and all the rats in the group fed the 10% diet died. Rats fed fungal-mat diets showed a marked reduction in weight gain, and the death rate was high (about 83%) in groups fed the 2 or 3% diet. Gross lesions found in groups fed either type of diet included focal necrosis in the liver, greenish discoloration of the kidneys, gastric ulceration, ulceration of the scrotal epidermis and corneal opacity. Histological changes in the liver included necrosis of the epithelium of biliary ducts, periductal oedema, pericholangitis, periductal fibrosis and disseminated focal hepatocellular necrosis. Necrosis of epithelium occurred also in the extraheptic ducts and was accompanied by an interstitial pancreatitis in certain rats. Foci of leucocytes and macrophages were found in the dermis of the scrotum and in the epididymal adipose connective tissue. Ulceration of the epidermis occurred late in the sequence of scrotal changes. Ocular lesions comprised corneal oedema, interstitital keratitis, iridocyclitis and hypopyon. Renal lesions consisted of tubular necrosis and hyaline and biliary casts within convoluted tubules.

INTRODUCTION

Aspergillus ochraceus has frequently been isolated from various foods, including heating grains (Wallace & Sinha, 1962), Japanese polished and unpolished rice (Udagawa, Ichinoe & Kurata, 1970), pepper (Christensen, Fanse, Nelson, Bates & Mirocha, 1967; Leyendecker, 1954), pecans (Doupnik & Bell, 1971) and Mexican corn (Lopez & Christensen, 1967), but has only been isolated infrequently from US stored corn. *A. ochraceus* may elaborate several mycotoxins, including ochratoxin A and penicillic acid (Ciegler, 1972; Hesseltine, Vandegraft, Fennell, Smith & Shotwell, 1972; Munro, Scott, Moodie & Willes, 1973; Natori, Sakaki, Kurata, Udagawa, Ichinoe, Saito & Umeda, 1970; van der Merwe, Steyn, Fourie, Scott & Theron, 1965) and these two mycotoxins may be produced singly or in combination not only by *A. ochraceus* but also by other members of the *A. ochraceus* group (Ciegler, 1972).

An isolate of *A. ochraceus* (Zimmermann, Carlton & Tuite, 1976) was found to be toxic when fed to mice, the hepatic lesions produced consisting of bile-duct necrosis, periductal inflammation and fibrosis and bile-duct hypertrophy and hyperplasia. Renal lesions comprised necrosis of the convoluted tubules and the presence of biliary casts within the convoluted tubules and collecting ducts. This spectrum of lesions was similar to that observed in mice and rats fed an Indiana isolate of *Penicillium viridicatum* (Budiarso, Carlton & Tuite, 1971; Carlton, Tuite & Mislivec, 1968 & 1970). This report describes the mycotoxicosis produced in rats by cultural products of an isolate of *A. ochraceus.*

EXPERIMENTAL

Rice cultures and fungal mats of *A. ochraceus* were prepared as previously described (Zimmermann *et al.* 1976) and mixed with a purified diet. Rice culture at dietary concentrations of 5, 7·5 and 10% and ground fungal mat at concentrations of 1, 2 and 3% were fed to groups of 12 male weanling Sprague-Dawley rats for a period of 5 wk. Control rats were fed a purified diet mixed with 10% rice treated with 1% propionic acid. Rats were housed in individual stainless-steel cages in an environmentally controlled room with the temperature maintained between 21 and 24°C. Water and feed were provided *ad lib.* The rats were weighed weekly. Rats that died, those killed when moribund and those killed when the experiments were terminated after 5 wk were autopsied. The liver, kidney, stomach, duodenum, pancreas, spleen and mesenteric lymph nodes, scrotum and eye were fixed in 10% buffered formalin, processed for paraffin sectioning, and stained with haematoxylin and eosin for histopathological examination.

RESULTS

Clinical signs

Diets containing rice culture or fungal mat were toxic to rats and resulted in reduced weight gains at all dietary concentrations and deaths in all but one group (Table 1). Rats fed the highest dietary concentration of rice culture (10%) did not survive beyond 2 wk, although there were few or no deaths in the groups fed the 7·5 and 5% concentrations. A high death rate was observed in groups fed the fungal-mat diets at concentrations of 2 or 3%, but few rats died in the group fed the 1% diet. Weight gains were markedly reduced in groups fed diets containing 2 or 3% fungal mat.

Rats fed either rice-culture or fungal-mat diets developed roughened hair, became anorectic and were dehydrated, and several from each dietary group developed grossly observable scrotal necrosis. By inspection, the LD_{50} for rats fed rice culture was determined to be between 7·5 and 10% and for those fed the fungal mat the LD_{50} was between 1 and 2%.

Gross pathology

Gross lesions in rats fed either rice culture or fungal mat were generally restricted to focal lesions in the liver, greenish discoloration of the kidneys and necrosis of the scrotal skin. Occasionally, rats fed either diet developed unilateral or bilateral corneal opacity. Early changes in the liver were a variation in colour from dark red to pale tan with accentuation of the lobular patterns. Rats autopsied after 7–10 days of feeding had variably-sized green pinpoint or larger foci throughout the various lobes. The majority were just under the capsule, although a few were visible on the cut surface. Gastric ulceration was found in a few rats fed either the fungal-mat or rice-culture diets (Table 2). These gastric lesions ranged from pinpoint craters to areas several millimeters in diameter within the glandular portion of the stomach. The mucosa surrounding the lesions was often swollen and was covered with considerable mucous. The majority of scrotal lesions occurred after 2 wk and were evident initially as dark areas about the midline accompanied by partial alopecia. Subsequently, small round to elliptical lesions followed the initial oedematous phase; these were often covered by a thin reddish scab which later became thickened and brownish-black. Additional foci developed, both laterally and posteriorly, and with expansion and coalescence the entire scrotal skin became necrotic. Occasionally linear areas of necrosis progressed anteriorly from the groin towards the flank, and rarely, small necrotic foci were found on the medial aspects of the thigh and on the tailhead. The penis and penile sheath were free of lesions. Most rats with necrosis of the scrotal skin also had a variable degree of epididymal fat necrosis. Grossly, involvement of the testes was not observed.

Gross ocular alterations occurred in three rats fed the rice-culture diets and in four fed the fungal-mat diets. These changes were characterized early by either unilateral or bilateral loss of corneal transparency and later by a slight greyish opacity of the cornea. Corneal changes usually began after 2 wk on either the rice-culture or fungal-mat diet and most regressed within 7–10 days.

Grossly, kidneys with lesions showed a greenish discoloration or were enlarged and pale.

Table 1. *Average weight gains and numbers of deaths in groups of 12 male rats fed a rice culture or fungal mat of* Aspergillus ochraceus

Test material and dietary level (%)	Mean body weight (g)		No. of deaths
	Initial	Final (wk 5)	
Rice culture			
0*	54	248	0
5	54	165	0
7.5	56	110	3
10	55	—†	12
Fungal mat			
0*	52	212	0
1	57	170	1
2	55	80	10
3	58	85	10

* Control rats were fed a purified diet mixed with 10% rice.

† No survivors at wk 5.

Microscopic pathology

The severity and numbers of lesions were more variable in rats fed the fungal-mat diets than in the groups fed the rice-culture diets. This variation was due in part to the early death of the rats fed the highest concentration of rice culture. The highest incidence and greatest severity of hepatic and renal lesions occurred in rats fed the two higher fungal-mat concentrations (Tables 3 and 4).

The earliest histological alterations in the liver consisted of variable numbers of mononuclear inflammatory cells within the portal areas and a proliferation of biliary epithelium, which formed short columns and aggregates of cells (Fig. 1). These changes occurred consistently in association with a necrotizing cholangitis characterized by flattening and necrosis of the epithelium of the intrahepatic bile ducts (Fig. 2). In some livers, the bile-duct necrosis was accompanied by periductal oedema and infiltration of the oedematous tissue by inflammatory cells (Fig. 3). In severely affected livers, most of the portal areas contained necrotic bile ducts and in some portal areas the portal vein and hepatic artery and the surrounding hepatic parenchyma were necrotic as well (Fig. 4). In occasional rats fed the 3% fungal-mat diet, necrotic foci had coalesced to produce areas of necrosis that incorporated several contiguous lobules. In rats that survived longer than 10 days, the bile-duct epithelium had proliferated and lined partially necrotic ducts, with an associated increase in fibrous connective tissue in the portal areas. Frequently some bile ducts were occluded by tissue debris, inflammatory cells and proliferating fibrous tissue, producing an obliterative cholangitis (Fig. 5). Although not commonly observed, mineralization was present in some of the larger foci of hepatocellular necrosis.

Alterations similar to those in the intrahepatic bile ducts were observed within the extrahepatic ducts, with the additional occurrence of thrombosis of the

lymphatic vessels. The extrahepatic ducts passing within the pancreas were often necrotic, and necrotizing and inflammatory changes commonly extended into the adjacent pancreatic tissue. The severity of the secondary pancreatitis was related to the severity of the involvement of the extrahepatic ducts. Pancreatitis was observed in all groups, but was most severe in animals fed the fungal-mat diets.

Hepatocellular alterations were minimal and were similar for rats fed the rice-culture or fungal-mat diets. Infrequently, vacuolation of hepatocytes was observed, tending to occur more commonly in rats that had been anorectic for an extended period.

Renal lesions in rats fed the rice-culture diets were mild and consisted only of hyaline casts within the cortical tubules. Rats fed the fungal-mat diets showed

Table 2. *Macroscopic lesions observed in male rats fed a rice culture or fungal mat of* Aspergillus ochraceus

Test material and dietary level (%)	No. of rats affected per group of 12						
	Liver		Kidney	Scrotum		Stomach	
	Focal necrosis	Enlarged extrahepatic ducts	Green discoloration	Necrosis	Epididymal steatitis	Blood in lumen	Mucosal ulceration
Rice culture							
5	3	1	1	10	6	0	0
7.5	9	1	4	10	7	3	1
10	10	0	0	1	1	3	0
Fungal mat							
1	4	0	2	9	8	0	0
2	11	3	3	5	5	4	1
3	11	2	3	3	3	4	0

Table 3. *Microscopic hepatic lesions observed in male rats fed a rice culture or fungal mat of* Aspergillus ochraceus

Test material and dietary level (%)	No. of rats (per group of 12) affected by			
	Multifocal necrosis	Necrotizing cholangitis	Pericholangitis	Extrahepatic-duct necrosis
Rice culture				
0	0	0	0	0
5	10	10	11	2
7.5	10	10	10	4
10	9	10	8	6
Fungal mat				
0	0	0	0	0
1	4	10	10	5
2	12	12	12	10
3	11	12	12	9

Table 4. *Microscopic renal lesions observed in male rats fed a rice culture or fungal mat of* Aspergillus ochraceus

Test material and dietary level (%)	No. of rats (per group of 12) affected by		
	Tubular casts		Tubular necrosis
	Hyaline	Biliary	
Rice culture			
0	0	0	0
5	3	0	0
7.5	0	0	0
10	0	0	0
Fungal mat			
0	0	0	0
1	2	0	4
2	0	2	4
3	1	0	4

tubular degeneration and necrosis (Fig. 6), hyaline and biliary casts within tubular lumens and the presence of biliary pigment within the cytoplasm of renal tubules (Table 4). The glomeruli appeared normal.

Gastric lesions were found in rats fed the fungal-mat diets or the 5 or 7·5% rice-culture diets (Table 5). Gastric alterations were characterized by necrosis extending for about one third to two thirds of the depth of the mucosa. The craters contained necrotic leucocytes, erythrocytes, fibrin and tissue debris (Fig. 7). In the gastric submucosa, leucocytic infiltrates were accompanied by fibrinoid necrosis of small arteries and veins. No ulceration or necrosis was found in the intestine.

Scrotal lesions occurred in rats fed the fungal-mat diets or the 5 or 7·5% rice-culture diets. They were not found in rats fed 10% rice culture as all these died within 2 wk of the start of feeding and scrotal alterations did not become evident macroscopically until later in the feeding period. The lesions in the grossly swollen scrota were numerous foci of leucocytes and macrophages or a diffuse infiltration of mixed inflammatory cells within the epididymal connective tissue (Fig. 8). Later, the overlying epithelium became necrotic and the dermis and subctuaneous adipose connective tissue contained leucocytes and macrophages. Frequently, the walls of arterioles and venules had undergone fibrinoid necrosis (Fig. 9). In some rats fed for a longer period, medium to large foci of mainly mononuclear inflammatory cells were found within the epididymal fat, the principal site of involvement being the anterior epididymal adipose connective tissue. The adipose tissue was infiltrated mainly by leucocytes and macrophages arranged in either irregular foci, trabeculae or bands. Often, the foci of leucocytes were necrotic and mineralized. In severely affected rats, the inflammatory exudate extended dorsally and laterally within the scrotal fascia and into the fibrous connective tissue about the epididymal ducts. The epididymal ducts were often affected by the advancing necrotizing reaction, resulting in periductal inflammation, necrosis of duct walls and accumulation of inflammatory cells within the ductal lumina (Fig. 10). No changes were noted either in the testicular interstitium or in the seminiferous tubules.

Corneal oedema was observed in some rats of all the test groups (Table 5). Interstitial keratitis occurred only in some rats fed the 2 or 3% fungal-mat diets. Iritis occurred in one rat fed the 7·5% rice-culture diet and in two fed the 3% fungal-mat diet (Table 5). The earliest ocular change was oedema of the corneal stroma and hydropic degeneration of the corneal epithelium. The substantia propria was thickened and showed only pale staining, and the lamellae were disrupted and separated by oedemal fluid (Fig. 11). In some eyes, the basal layers of the corneal epithelium were separated by mild intercellular oedema, while in those more severely affected the substantia propria was diffusely infiltrated by neutrophils. Neocapillary vascularization was an infrequent finding. Iridocyclitis and hypopyon accompanied the acute interstitial keratitis in some eyes. The exudate in the anterior chamber consisted on neutrophils and mononuclear cells with formation of small keratic precipitates. Iridocyclitis was characterized by an accumulation of neutrophils within the stroma of the iris and ciliary body. In eyes with severe iridocyclitis, the iris was adherent to the lens capsule (Fig. 12).

DISCUSSION

Development of lesions in the liver of rats fed diets containing a rice culture or fungal mat of *A. ochraceus* followed a fairly definite pattern. Early, hypertrophy and hyperplasia of the bile-duct epithelium was accompanied by proliferation of the bile ducts. Later, a necrotizing cholangitis was associated with extension of the necrosis into the surrounding hepatic parenchyma, with periductal fibrosis and with stenosis and obliteration of the bile ducts. The hepatic lesions were identical to those produced in mice and rats by cultural products of *P. viridicatum* (Budiarso *et al.* 1971; Carlton *et al.* 1968 & 1970; McCracken, Carlton & Tuite, 1974c) and in mice by cultural products of *A. ochraceus* (Zimmermann *et al.* 1976). The morphological pattern of the hepatic lesions in rats and mice suggested that the lesion began in the

Table 5. *Microscopic lesions observed in the scrotum, eye, stomach and pancreas of rats fed a rice culture or fungal mat of* Aspergillus ochraceus

Test material and dietary level (%)	No. of rats affected per group of 12						
	Scrotum		Eye			Stomach	Pancreas
	Epididymal steatitis/ epididymitis	Epidermitis/ dermatitis	Iritis	Corneal oedema	Keratitis	Ulceration	Pancreatitis
Rice culture							
0	0	0	0	0	0	0	0
5	7	8	0	1	0	1	0
7.5	6	8	1	1	0	1	0
10	0	0	0	7	0	0	3
Fungal mat							
0	0	0	0	0	0	0	0
1	4	8	0	1	0	3	2
2	5	7	0	5	1	2	7
3	4	5	2	7	2	2	5

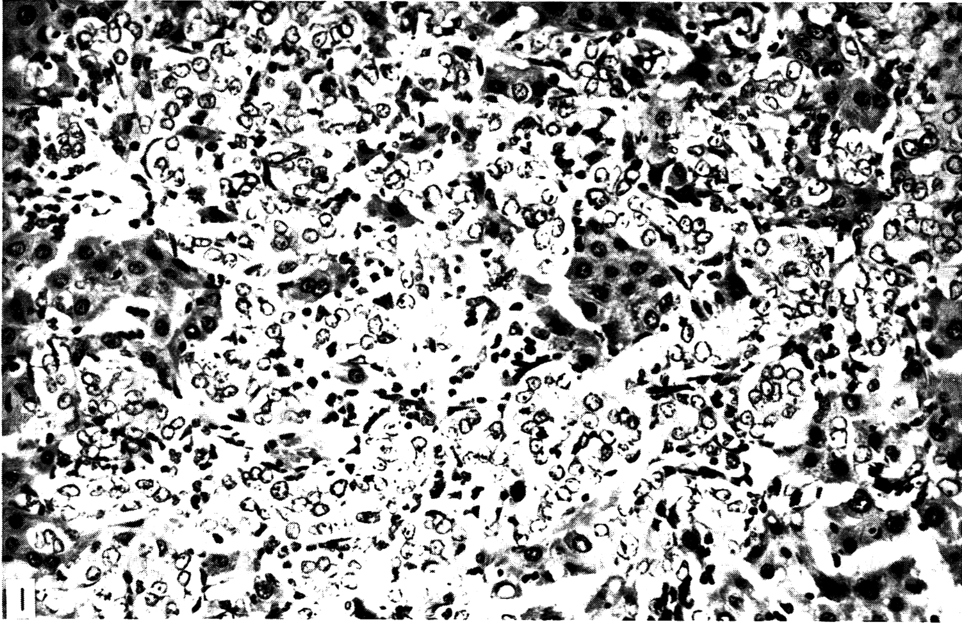

Fig. 1. Hyperplasia of biliary epithelium resulting in the formation of short columns and aggregates of cells. Haematoxylin and eosin (H/E) × 228.

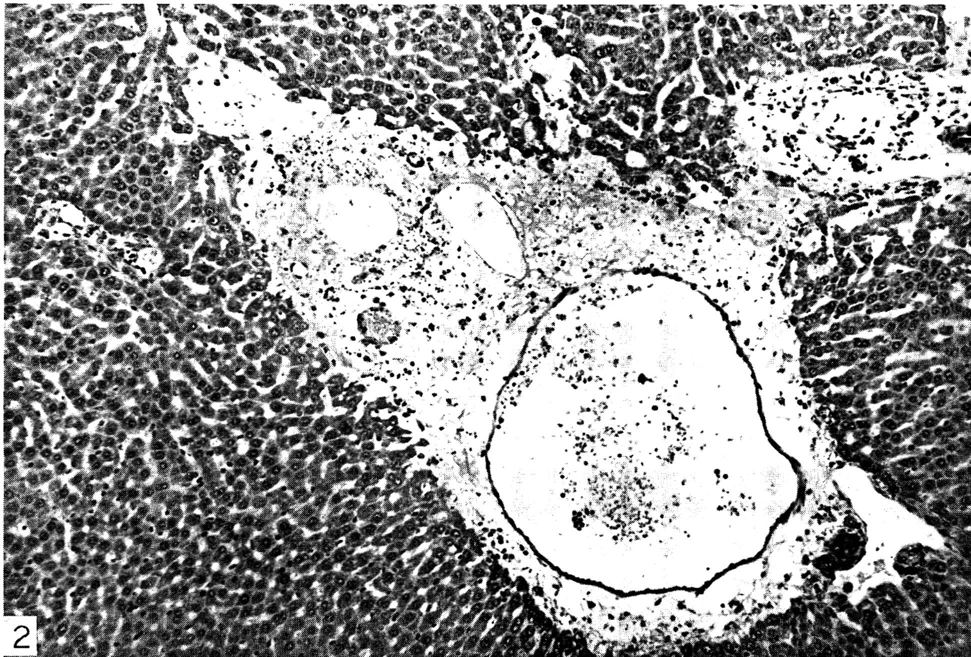

Fig. 2. Necrotizing cholangitis, characterized by necrosis centered about the portal triads. H/E × 88.

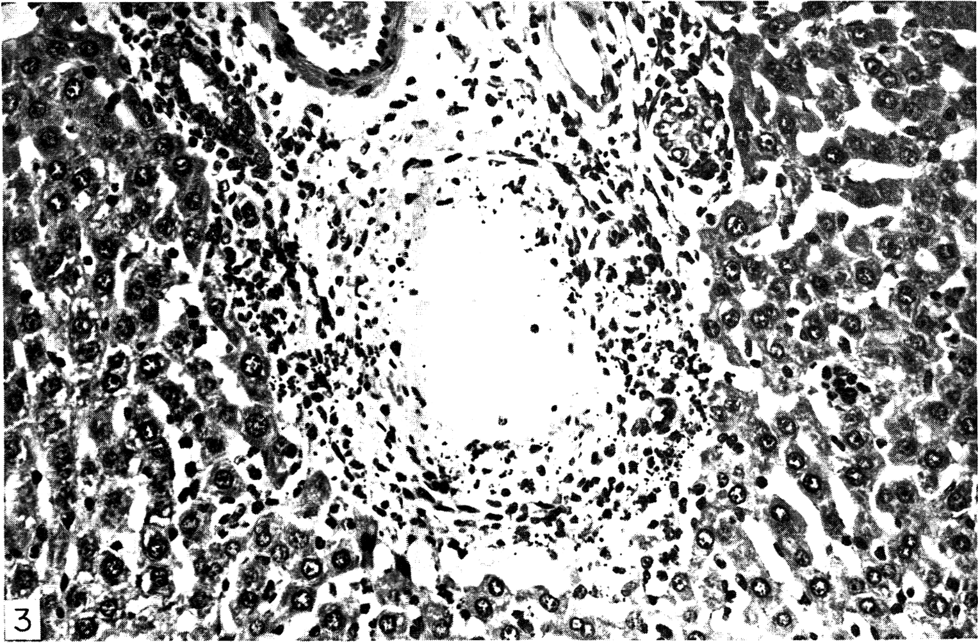

Fig. 3. Necrosis of intrahepatic bile ducts accompanied by periductal oedema and infiltration of the oedematous tissue with inflammatory cells. H/E × 228.

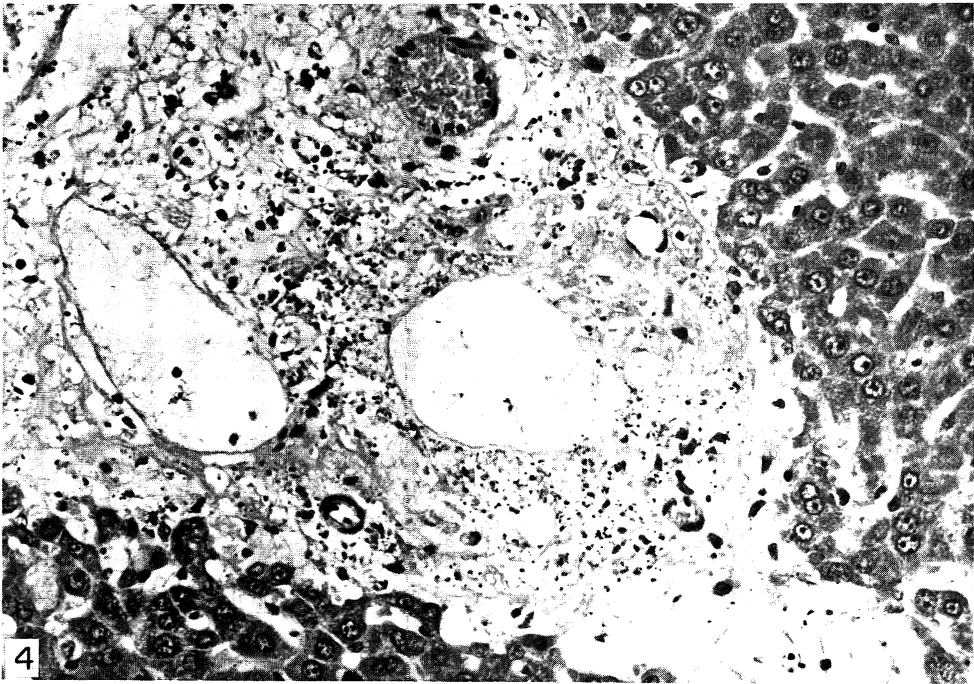

Fig. 4. Necrosis of portal structures, including bile ducts, the portal vein and hepatic artery and adjacent parenchyma. H/E × 228.

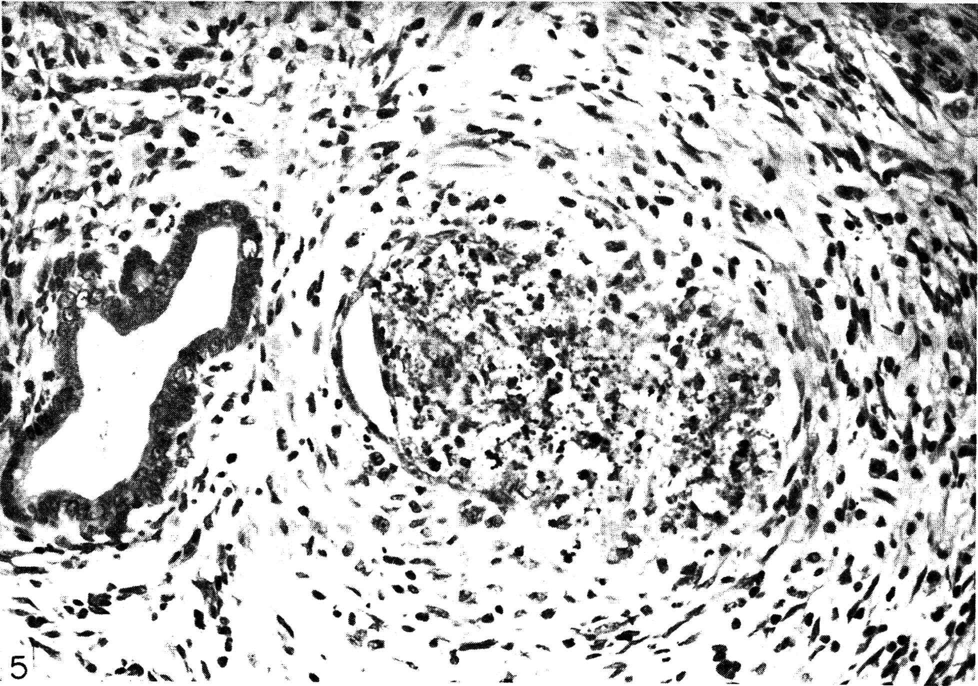

Fig. 5. Occlusion of bile duct by tissue debris and inflammatory cells, accompanied by periductular proliferation of fibrous connective tissue. H/E × 228.

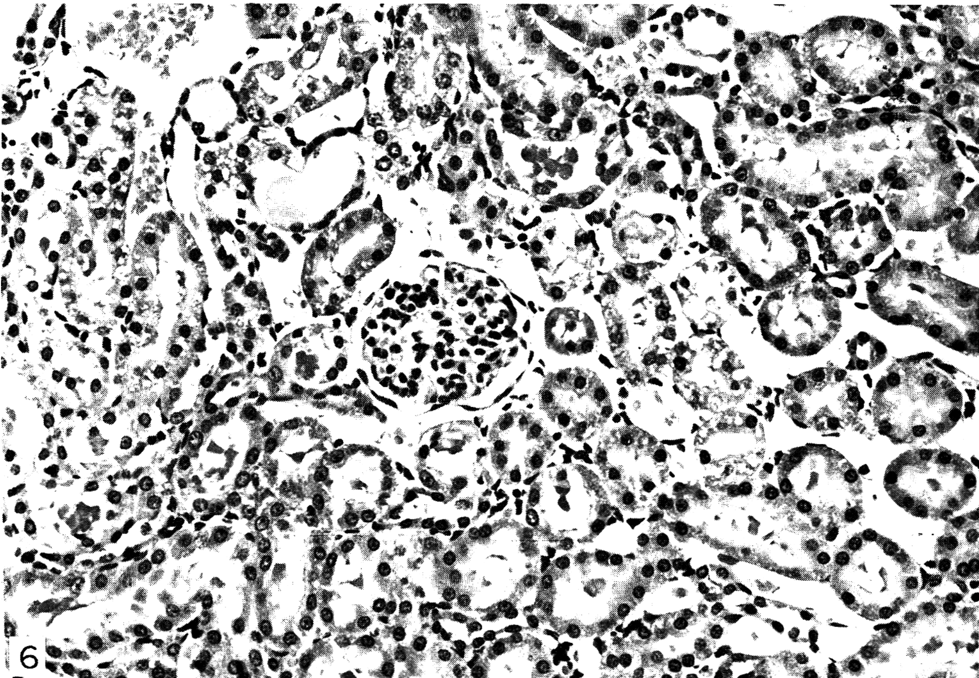

Fig. 6. Renal tubular degeneration with hyaline casts and a normal glomerulus. H/E × 228.

Fig. 7. Gastric erosion containing fibrin, tissue debris and necrotic epithelium, accompanied by submucosal cellulitis. H/E × 88.

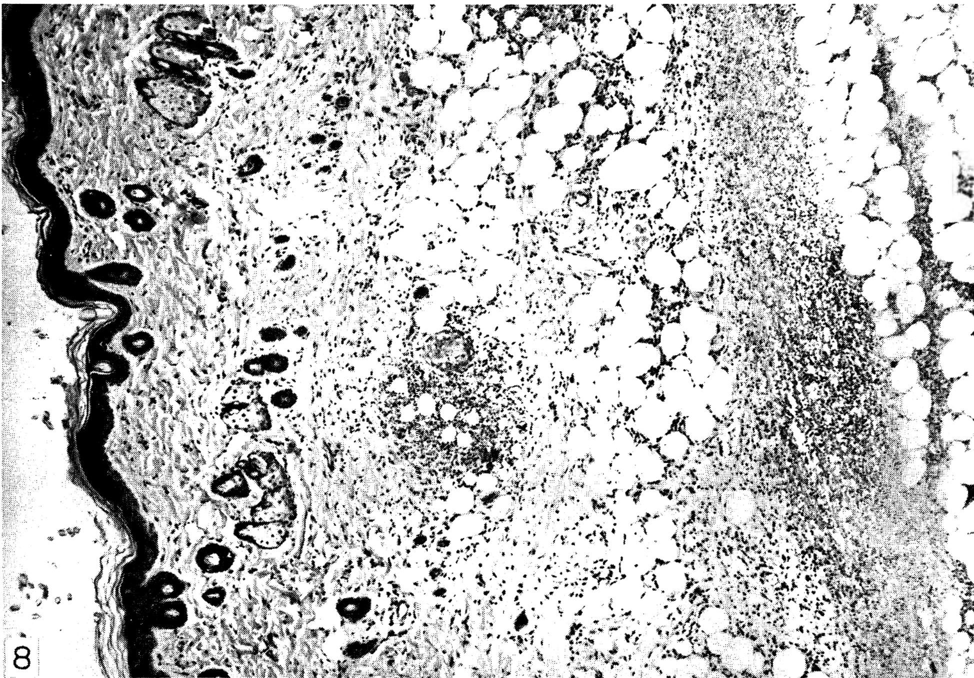

Fig. 8. Infiltration of inflammatory cells into the adipose tissue of the scrotal dermis. H/E × 56.

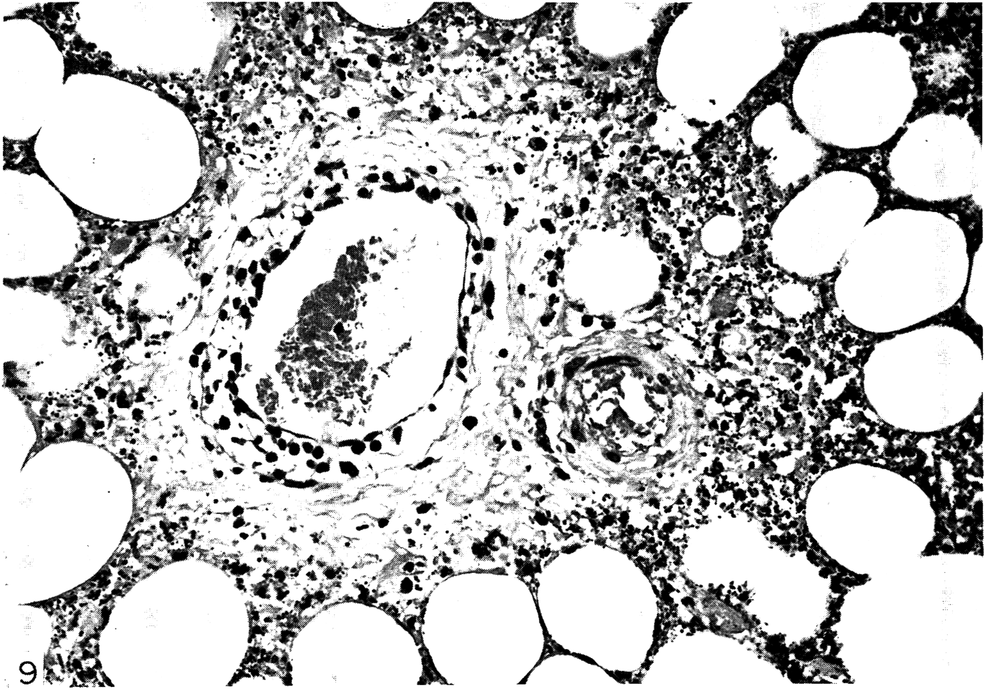

Fig. 9. Fibrinoid necrosis of an arteriole and phlebitis of an adjacent venule within the scrotal adipose connective tissue. H/E × 228.

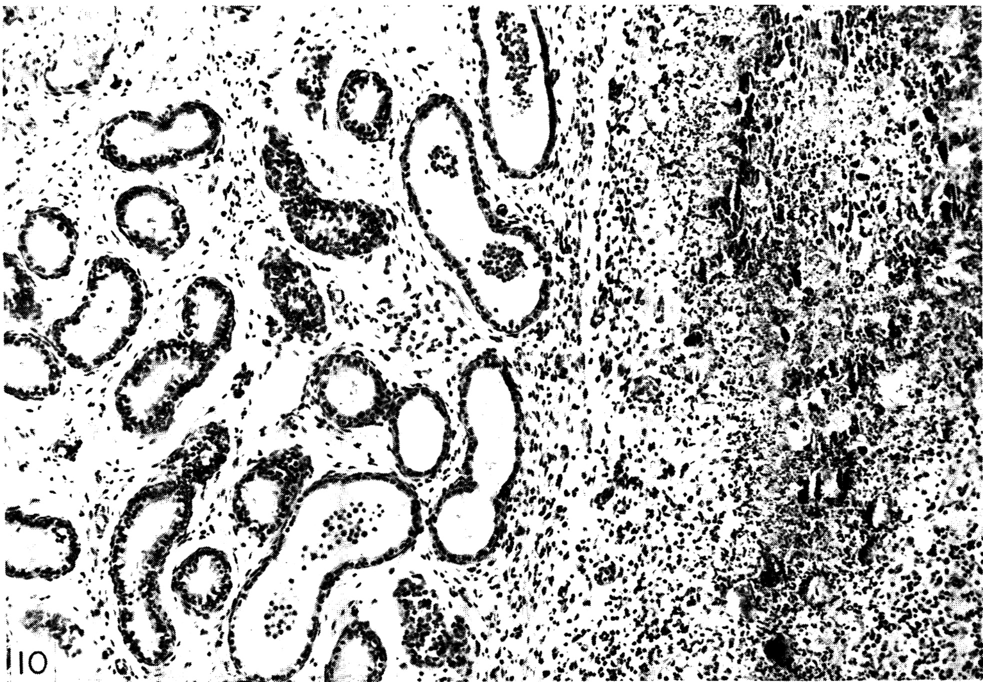

Fig. 10. Inflammation and necrosis of the epididymal adipose tissue, accompanied by an accumulation of inflammatory cells within the ductal lumina. H/E × 88.

Fig. 11. Corneal oedema, hydropic degeneration of the epithelium and a mild infiltration of the substantia propria with a mixture of inflammatory cells. H/E × 228.

Fig. 12. An iris infiltrated with inflammatory cells and adhering to the anterior lens capsule. H/E × 228.

intrahepatic bile ducts and that the necrosis of the surrounding tissue was the result of the damage to the biliary system, allowing leakage of bile or toxin(s) into the surrounding hepatic parenchyma.

The hepatic lesions observed in these rats showed some similarity to lesions induced in rats by sporidesmin (Remington, Slater, Spector, Strauli & Willoughby, 1962). However, the pleural effusions and ascites that were prominent findings in sporidesmin poisoning were not seen in the rats of this study. Other hepatotoxic mycotoxins do not produce a necrotizing cholangitis or cholangiohepatitis and most are characterized by hepatic degeneration and necrosis, sometimes accompaneid by bile-duct hyperplasia. Such mycotoxins include the aflatoxins (Wogan, 1965), luteoskyrin (Kobayashi, Uraguchi, Sakai, Tatsuno, Tsukioka, Sakai, Sato, Miyake, Saito, Enomoto, Shikata & Ishiko, 1958; Uraguchi, Tatsuno, Sakai, Tsukioka, Sakai, Yonemitsu, Ito, Miyake, Saito, Enomoto, Shikata & Ishiko, 1961), cyclopiazonic acid (Purchase, 1971), the epoxytrichothecenes (Saito, Enomoto & Tatsuno, 1969) and rubratoxin A produced by certain strains of *P. rubrum* (Wogan, Edwards & Newberne, 1971). Ochratoxin A toxicosis results in non-specific hepatocellular degeneration and necrosis. However, this particular mycotoxin has not been found in our isolate of *A. ochraceus*. Several species of Aspergilli can produce sterigmatocystin, which causes focal hepatic necrosis, proliferation of bile ducts, pericholangitis and hyperplastic nodules (Purchase & van der Watt, 1969). Of the mycotoxins, then, only the sporidesmins (and the pigments xanthomegnin and viomellein) elicit tissue responses similar to those observed with *A. ochraceus* and *P. viridicatum*. Metabolites with chemical characteristics consistent with the sporidesmins have not been isolated from cultures of *A. ochraceus* or *P. viridicatum*.

Gastric ulceration and varying degrees of submucosal cellulitis were present in some of our rats. Necrosis of the epithelium of the digestive tract and, in particular, of the crypt epithelium has been observed in rats and mice given extracts of *Fusarium nivale* (Tatsuno, 1968) and with the purified mycotoxins nivalenol (Tatsuno, Saito, Enomoto & Tsunoda, 1968) and fusarenon-X (Ueno, Ueno, Iitoi, Tsunoda, Enomoto & Ohtsubo, 1971). Ulceration of the intestinal mucosa and necrosis of the crypt epithelium were not observed in our rats fed *A. ochraceus*.

Scrotal lesions similar to those observed in our rats fed cultural products of *A. ochraceus* have been described in rats given cultural products of isolates of *P. viridicatum* (Carlton & Tuite, 1970; McCracken, Carlton & Tuite, 1974b; Rafiquzzaman, 1974). Budiarso, Carlton & Tuite (1970) described testicular lesions in mice fed *P. viridicatum*. No set of similar scrotal lesions has been reported in the rat as a result of either an infection or a mycotoxicosis. Scrotal lesions in our rats began and were most severe and numerous in the epididymal fat. At an early stage, mononuclear cells infiltrated the dermis and the epididymal fat and these infiltrates formed discrete accumulations of leucocytes and macrophages frequently oriented about blood vessels. Later, bands of necrotic leucocytes and macrophages transsected the junction between the dermis and subcutis with obliteration of large portions of the epididymal adipose connective tissue. The epidermis was not consistently involved and most often seemed to be secondarily affected at a late stage of the cellulitis. The development of lesions deep within the scrotal tissue, many centering about blood vessels, and the lack of concomitant involvement of the epidermis indicated that the changes were the result of circulating toxin(s) and not due to surface contamination with toxic diet. Several of the trichothecenes, such as diacetoxyscirpenol, T-2 toxin, HT-2 toxin, nivalenol and fusarium-X produced by several species of Fusarium and other fungal genera, are highly toxic when applied topically to the skin of rats, mice and rabbits, but cutaneous lesions have not been described following oral or parenteral administration (Bamburg & Strong, 1971).

The cutaneous scrotal lesions in our rats were initially multiple foci of inflammation and necrosis within the subcutis, progressing subsequently to epidermal ulceration. McCracken *et al.* (1974b) described similar alterations in rats fed cultural products of *P. viridicatum* and suggested that the lesions within the scrotal adipose connective tissue were due to some blood-borne toxin(s). By sequential killing of the rats, McCracken *et al.* (1974b) demonstrated a definite association between inflammatory cell infiltration about blood vessels, vascular thrombosis and epidermal necrosis. In our rats, the epithelium of the epididymal ducts and seminiferous tubules was not primarily altered, but testicular lesions have been reported with a number of other mycotoxins, including extracts of *F. nivale*, nivalenol (Ueno *et al.* 1971) and neosolanil (Ishii, Sakai, Ueno, Tsunoda & Enomoto, 1971; Ueno, Ishii, Sakai, Kanaeda, Tsunoda, Tanaka & Enomoto, 1972). The necrotizing scrotal lesions appear to be a unique response of rats to cultural products of *A. ochraceus* and *P. viridicatum* as they were not found in guinea-pigs (Carlton & Tuite, 1970) or mice (Budiarso *et al.* 1970) fed *P. viridicatum*, and have not been described in other mycotoxic diseases including those caused by aflatoxin (Wogan, 1965), ochratoxin A (Purchase & Theron, 1968), rubratoxin (Wogan *et al.* 1971) and sterigmatocystin (van der Watt & Purchase, 1970).

Ocular lesions developed in most rats fed rice-culture or fungal-mat diets. The earliest alteration was corneal oedema and this was often followed by interstitial keratitis and hypopyon. Because normal hydration and transparency of the corneal stroma is dependent upon the balance between the influx of fluid into and the active transport of fluid out of the stroma (Dikstein & Maurice, 1972; Mishima & Hayakawa, 1972) and because the control mechanism for this resides in the corneal endothelium (Dikstein & Maurice, 1972; Mishima & Hayakawa, 1972), it seems possible that circulating toxin(s) might be present in the anterior chamber, resulting in damage to the corneal endothelium. McCracken, Carlton & Tuite (1974a) described similar ocular lesions in rats fed cultures of *P. viridicatum*. Several fungal extracts and mycotoxins have produced corneal opacity when applied topically into the conjunctival sac of rabbits. These include purified sporidesmin and extracts of *Pithomyces chartarum* (Done, Mortimer, Taylor & Russell, 1961), patulin (Broom, Bulbring, Chapman,

Hampton, Thomson, Ungar, Wein & Woolfe, 1944) and diacetoxyscirpenol (Brian, Dawkins, Grove, Hemming, Lowe & Norris, 1961), but none of these are known to produce ocular lesions following oral or parenteral administration.

Bacteria were not detected in swabs from the interior of the eyes of rats fed the rice culture or fungal mat. Two rats had inflammatory changes of the Harderian gland consistent with the changes described for sialodacryoadenitis of rats caused by a corona virus (Hunt, 1963; Jacoby, Bhatt & Jonas, 1975; Jonas, Craft, Black, Bhatt & Hilding, 1969). These Harderian gland lesions were incidental and occurred in rats with and without lesions of the cornea and anterior uvea.

A toxin has not been recovered in pure form from our strain of *A. ochraceus*. Analyses carried out by one of us (J.T.) in the Department of Botany and Plant Pathology have shown the cultures to be negative for aflatoxin, ochratoxin A, citrinin and penicillic acid. Stack, Eppley, Dreifuss & Pohland (1977) isolated viomellein and xanthomegnin pigments from an Indiana isolate of *P. viridicatum*, which produced hepatic lesions in mice and rats identical to those induced by our strain of *A. ochraceus*. These pigments, xanthomegnin and viomellein, incorporated into a purified diet and fed to mice, produced hepatic lesions identical to those produced by cultural products of *P. viridicatum* (Carlton, Stack & Eppley, 1976). Experiments are currently being conducted to determine whether these pigments and others are also present in cultural products of *A. ochraceus*.

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CONVERSION OF RUBRATOXIN B TO THE CARBOXYLIC ACID DERIVATIVE AND ITS EFFECT ON ADENOSINE TRIPHOSPHATASE ACTIVITY AND TOXICITY TO MICE

P. D. UNGER, T. D. PHILLIPS and A. W. HAYES
Department of Pharmacology and Toxicology, University of Mississippi Medical Center, Jackson, MS 39216, USA

(*Received 25 March 1978*)

Abstract—The rate of conversion of rubratoxin B to its carboxylic acid derivative upon exposure to aqueous solvents was exponentially related to the relative abundance of water molecules. The same relationship existed for the rate of decrease in inhibition of Na^+-K^+ adenosine triphosphatase (ATPase) activity by rubratoxin B, when the toxin was incubated in the reaction mixture prior to addition of the enzyme. Inhibition of this enzyme activity by rubratoxin B was dose-dependent, with an IC_{50} of $5{\cdot}8 \times 10^{-6}$ M, while the carboxylic acid derivative was much less effective, the IC_{50} being $19{\cdot}3 \times 10^{-6}$ M. In mice, the ip LD_{50} of the carboxylic acid derivative dissolved in propylene glycol was more than twice that of the parent compound 24 hr after dosing, but beyond 24 hr there was little difference between the two values.

INTRODUCTION

Rubratoxin B (Fig. 1), a potent mycotoxin produced by *Penicillium rubrum*, has been isolated from a variety of agricultural products, including cereals and legumes (Blevins, Glenn, Hamdy, Brodasky & Evans, 1969; Scott, 1965), peanuts (Joffe & Borut, 1966) and maize, bran and sunflower seeds (Cantini & Scurti, 1965). The toxic effects attributed to rubratoxin B include hepatotoxicity (Burnside, Sippel, Forgacs, Carll, Atwood & Doll, 1957), mutagenicity (Evans & Harbison, 1977) and teratogenicity (Hood, Innes & Hayes, 1973).

Formation of artefacts is a problem in all the chromatographic techniques developed for the identification and quantitation of rubratoxin B. Hayes & Wilson (1968) observed a polar artefact in a silica-gel thin-layer chromatographic technique for rubratoxin B, while Moss & Hill (1970) observed that co-chromatography of rubratoxins A and B resulted in four rather than two spots. Hayes & McCain (1975) noted that the artefact was eliminated when thin-layer plates were spotted and developed in an atmosphere of nitrogen, hydrogen or oxygen. A polar artefact has also been observed in a high-pressure liquid chromatography (HPLC) procedure for rubratoxin, using acetonitrile–water–glacial acetic acid (55:45:2, by vol.) as the elution solvent (Engstrom, Richard & Cysewski, 1977). Rubratoxin B behaves as an acid in aqueous solution (Moss, Robinson & Wood, 1971) and absorbance characteristic of carboxylic acids has been detected in the infra-red spectrum of rubratoxin B (Townsend, Moss & Peck, 1966). The artefacts, therefore, almost certainly result from hydrolysis of the maleic anhydride rings in the rubratoxin molecule (Moss, 1971).

Recent results from this laboratory indicated that rubratoxin B was rapidly and reversibly converted to the carboxylic acid derivative upon exposure to aqueous solvents (Unger & Hayes, 1978). Concurrently it was noted that addition of rubratoxin B to aqueous reaction mixtures prior to the addition of enzyme resulted in a loss of inhibition of Na^+-K^+ adenosine triphosphatase (ATPase) activity. Since biological fluids are primarily aqueous, the present work was undertaken to study the conversion of rubratoxin B to its carboxylic acid derivative and the effects of the conversion on one biological system, ATPase activity. Inhibition of ATPase by rubratoxin B *in vivo*, demonstrated by Desaiah, Hayes & Ho (1977) may be a biochemical mechanism for the toxic action of the compound. The LD_{50} of rubratoxin B when administered ip to mice was therefore compared with that of the carboxylic acid derivative.

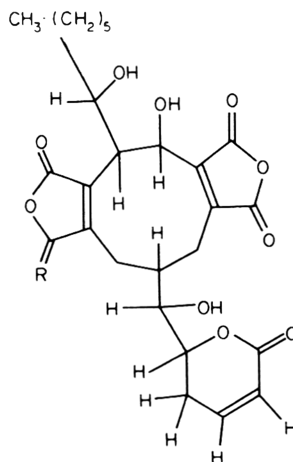

Fig. 1. Structure of the rubratoxins; for rubratoxin A ($C_{26}H_{32}O_{11}$) R = H, OH, and for rubratoxin B ($C_{26}H_{30}O_{11}$) R = O. In aqueous solution the maleic anhydride moieties on either side of the nine-membered ring equilibrate with the open form to yield a carboxylic acid derivative.

EXPERIMENTAL

Preparation of rubratoxin B and the carboxylic acid derivative. Rubratoxin B was prepared according to the procedure of Hayes & Wilson (1968). Its purity was confirmed by infra-red absorbance, HPLC, melting point and mass-spectral analysis. The carboxylic acid derivative was prepared by exposing rubratoxin B dissolved in acetonitrile (1 mg/ml) to 20 vols deionized glass-distilled water for 3 hr; the solution was then freeze-dried and the lyophilizate was used for infra-red absorbance spectroscopy, ATPase assay and the LD_{50} study.

Infra-red absorbance. Mixtures of KBr with 3% freeze-dried material or authentic rubratoxin B were dried at 100°C for 5 min prior to being pressed into pellets for the determination of infra-red absorbance spectra.

High-pressure liquid chromatography. Reverse-phase HPLC was carried out as previously described (Unger & Hayes, 1978). Separation of rubratoxin B from the carboxylic acid derivative was achieved using a μ-Bondapak C18 column (Waters Associates, Inc., Milford, MA) with detection at 254 nm. Volumes of 50 μl acetonitrile containing 50 μg of rubratoxin B were mixed with 50, 100, 150 or 200 μl glass-distilled deionized water to give $2{\cdot}88 \times 10^4$, $5{\cdot}76 \times 10^4$, $8{\cdot}63 \times 10^4$ and $11{\cdot}51 \times 10^4$ mol water/mol rubratoxin B, respectively. For each of these mixtures, aliquots containing 1 μg rubratoxin B were injected at timed intervals of approximately 3·5 min and the areas of the peaks of rubratoxin B were measured.

ATPase studies. Two ATPase preparations, a microsomal fraction isolated from mouse brain as previously described (Phillips & Hayes, 1977) and a highly specific Na^+-K^+ ATPase from pig-cerebral cortex microsomes (Sigma Chemical Co., St. Louis, MO) were used. The former was used to examine the effects of rubratoxin B and its carboxylic acid derivative on Na^+-K^+ ATPase and basal Mg^{++} ATPase (oligomycin-sensitive and -insensitive components), and the latter, which was devoid of Mg^{++} ATPase, to determine the effects of rubratoxin B on Na^+-K^+ ATPase after pre-incubation of the toxin in the reaction mixture in the absence of enzyme. A 1-ml reaction mixture contained 5·0 mM-ATP, 5·0 mM-Mg^{++}, 100 mM-Na^+, 20 mM-K^+, 135 mM-imidazole.HCl (pH 7·5) and 50 μg enzyme protein. In the mouse-brain ATPase experiments, rubratoxin B, the carboxylic acid derivative of rubratoxin B, a propylene glycol control (solvent vehicle, 1 μl) and reagent and enzyme blanks were incubated simultaneously at 37°C for 10 min prior to initiation of the reaction with ATP. The reaction was stopped after 10 min by addition of trichloroacetic acid (TCA) at a final concentration of 5% w/v. ATP hydrolysis was linear with both enzyme preparations under the experimental conditions used. ATPase activities were measured by the endpoint phosphate analysis, as previously described (Lowry & Lopez, 1946). A standard inorganic phosphate curve was prepared for each experiment and extrapolated values were used to calculate specific activity (μmol P_i/mg protein/hr). Protein was determined by the method of Lowry, Rosebrough, Farr & Randall (1951) using bovine serum albumin as the standard.

In the pre-incubation study, 1·0 μl propylene glycol containing rubratoxin B (10–120×10^{-6} M in final concentration) was delivered, using a Hamilton microliter syringe, to the side of a test-tube containing the incubation mixture lacking only the enzyme. The hydrolysis reaction was initiated at timed intervals

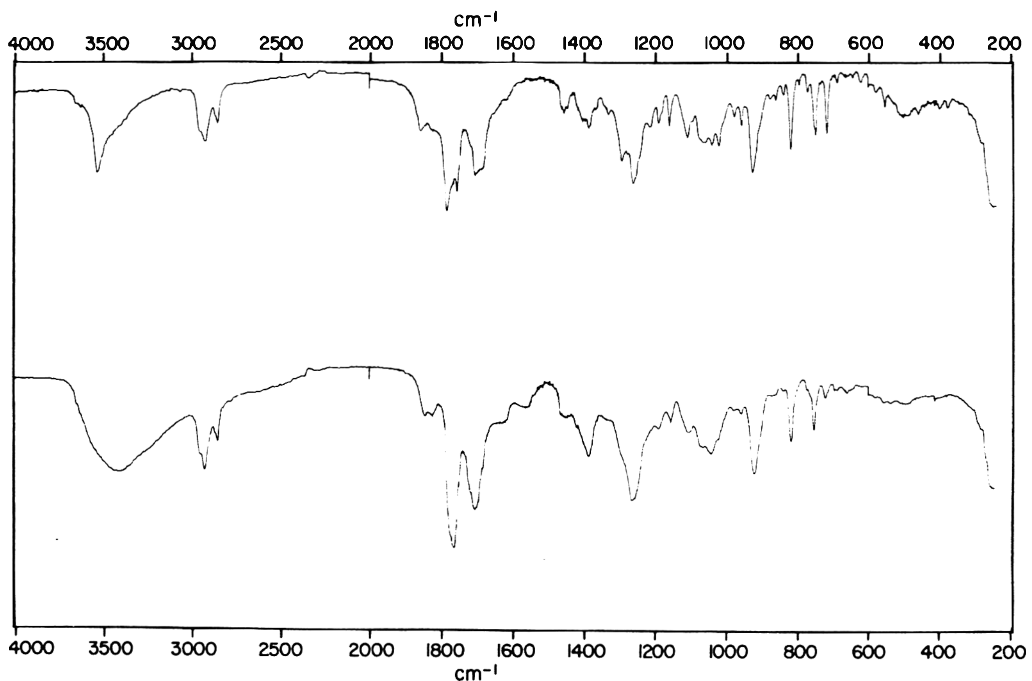

Fig. 2. Infra-red spectra of (above) rubratoxin B and (below) its carboxylic acid derivative in KBr discs.

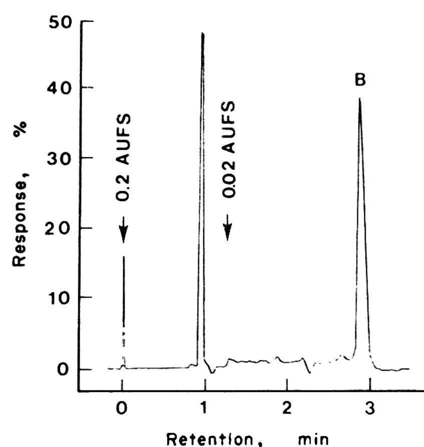

Fig. 3. HPLC chromatogram of a 3-μl aliquot of a mixture of 50 μg rubratoxin B in 50 μl acetonitrile and 100 μl water after incubation for 32 min.

carboxylic acid derivative at 1710 cm^{-1} indicates the presence of carboxylic acid functional groups. The broad absorbance band between 3000 and 3725 cm^{-1} could be due to polymeric OH association of the carboxylic acid derivative. The presence of some absorbance of the authentic compound at 1710 cm^{-1} is probably due to anhydride hydrolysis caused by atmospheric humidity.

It was possible to follow the conversion of rubratoxin B directly by HPLC, since in the reverse-phase chromatography system the carboxylic acid derivative eluted at 58 sec compared to 175 sec for the parent compound (Fig. 3). The loss in UV absorbance at 254 nm upon conversion of rubratoxin B to the carboxylic acid derivative was the result of the opening of the disubstituted maleic anhydride rings. The curves in Fig. 4 show that loss of UV absorbance of rubratoxin B upon exposure to water followed first-order kinetics and was biphasic with respect to time. The slow (β) phase of the curves probably

after mixing by addition of enzyme. The reaction was terminated after 10 min and then assayed for inorganic phosphate as described above.

LD_{50} study. Groups of five male ICR mice (20–30 g; Charles River, Wilmington, MA) were injected ip with a propylene glycol solution of authentic rubratoxin B or its carboxylic acid derivative in single doses of 0·36, 0·72, 1·41, 2·82 and 5·64 mg/kg, serially diluted so that each animal received the same volume of solvent vehicle. This volume of propylene glycol was injected ip into five additional animals which served as controls.

RESULTS

The infra-red absorbance spectra obtained from rubratoxin B (upper tracing) and its carboxylic acid derivative (lower tracing) are compared in Fig. 2. Less absorbance is indicated in the infra-red spectrum of the carboxylic acid derivative at 1790 and 1860 cm^{-1} indicating a decrease in the quantity of anhydride present. Increased absorbance of the spectrum of the

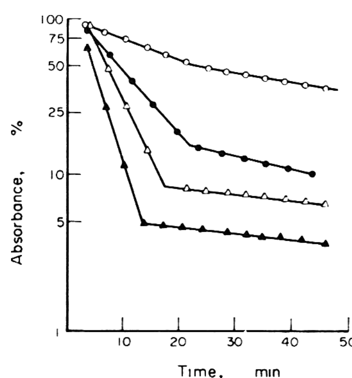

Fig. 4. Semi-log plot of rubratoxin B absorbance at 254 nm against time of exposure to water in a proportion of 2·88 × 10^4 (○), 5·76 × 10^4 (●), 8·63 × 10^4 (△) or 11·51 × 10^4 (▲) mol H_2O/mol rubratoxin B. Absorbance of the rubratoxin B peak of 1 μg rubratoxin B standard was taken as 100%. The points in the absorbance curves are means of values fitted to $y = ae^{\alpha x} + be^{\beta x}$ ($0·96 \leq r \leq 0·998$).

Table 1. *Effects of rubratoxin B and its carboxylic derivative on* Na^+-K^+ *and* Mg^{++} *ATPase activities* in vitro

Concn of toxin (10^{-6} M)	Rubratoxin B: Na^+-K^+ ATPase†	Rubratoxin B: Mg^{++} ATPase OS	Rubratoxin B: Mg^{++} ATPase OI	Carboxylic acid derivative: Na^+-K^+ ATPase‡	Carboxylic acid derivative: Mg^{++} ATPase OS	Carboxylic acid derivative: Mg^{++} ATPase OI
0§	42·5 ± 1·2	5·2 ± 1·1	16·8 ± 1·7	42·5 ± 1·2	5·2 ± 1·1	16·8 ± 1·7
1·0	38·3 ± 1·5	4·2 ± 2·0	16·5 ± 0·8	41·0 ± 0·6	5·4 ± 2·1	17·1 ± 2·3
2·0	34·4 ± 2·0*	3·6 ± 1·0	16·0 ± 2·1	39·9 ± 3·1	5·1 ± 1·3	16·5 ± 1·8
4·0	27·6 ± 2·1*	3·1 ± 1·3	15·6 ± 2·0	38·3 ± 1·2	5·6 ± 1·2	16·7 ± 1·7
6·0	18·7 ± 1·5*	2·6 ± 1·3*	15·1 ± 1·1	34·0 ± 1·7*	5·0 ± 2·3	17·0 ± 1·0
10·0	7·7 ± 0·9*	1·0 ± 1·5*	12·6 ± 1·5*	31·8 ± 2·3*	4·3 ± 1·7	16·0 ± 2·1

Specific activity (μmol P_i/mg enzyme protein/hr) of mouse-brain microsomal fraction in the presence of the toxin.

OS = Oligomycin-sensitive ATPase OI = Oligomycin-insensitive ATPase
†Linear regression analysis indicates $r = 0·99$.
‡Linear regression analysis indicates $r = 0·974$.
§Control: propylene glycol (1 μl) without rubratoxin B or carboxylic acid derivative.
Values are means ± SEM for two independent replicates each assayed in triplicate. Those marked with an asterisk differ significantly ($P < 0·05$ by Student's t test) from the corresponding propylene glycol-treated control value.

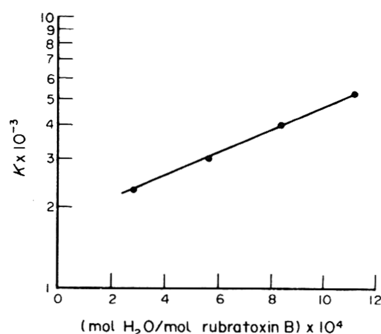

Fig. 5. Semi-log plot of first-order rate constants (K) of fast phase of curves shown in Fig. 4 against mol H_2O/mol rubratoxin B.

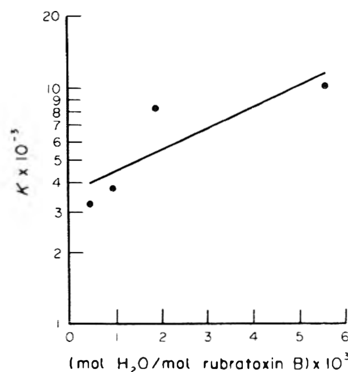

Fig. 7. Semi-log plot of first-order rate constants (K) of curves shown in Fig. 6 against mol H_2O/mol rubratoxin B.

represents equilibration between the open and closed ring forms, biased slightly in favour of the open form. The curves were feathered to determine the true α (slope of the fast phase) and a rate constant K was calculated for each curve. When the K values were plotted semi-logarithmically against the number of moles of water divided by the number of moles of rubratoxin B added (mol H_2O/mol rubratoxin B), a straight line resulted ($r > 0{\cdot}999$) with a slope of $1{\cdot}004 \times 10^{-5}$ (Fig. 5). This finding indicated an exponential relationship between the rate of conversion of rubratoxin B to the carboxylic acid derivative in the fast phase of the reaction and the relative abundance of water molecules.

Inhibition of mouse-brain microsomal Na^+–K^+ ATPase by rubratoxin B and the carboxylic acid derivative was linear through a concentration range of 0–10 × 10^{-6} M (Table 1). The concentration of rubratoxin B resulting in 50% inhibition of Na^+–K^+ ATPase (IC_{50}) was $5{\cdot}8 \times 10^{-6}$ M, whereas the IC_{50} for the carboxylic acid derivative was more than three times as great ($19{\cdot}3 \times 10^{-6}$ M). The data in Table 1 also indicate that the basal Mg^{++} ATPase activity was inhibited by rubratoxin B (the oligomycin-sensitive portion more than the oligomycin-insensitive). However, the carboxylic acid derivative at concentrations up to 10×10^{-6} M showed no significant effects on either the oligomycin-sensitive or -insensitive Mg^{++} ATPase activities suggesting complete loss of toxicity to these enzyme components (Table 1).

Inhibition of Na^+–K^+ ATPase activity by rubratoxin B was independent of pre-incubation time when enzyme was present in the reaction mixture prior to toxin addition. However, incubation of rubratoxin B in the reaction mixture prior to enzyme addition resulted in a rapid time-dependent reduction of inhibition (Fig. 6). The loss of inhibition of Na^+–K^+ ATPase activity followed monophasic first-order kinetics to 60 sec. When the first-order rate constants (K) of each curve were plotted semi-logarithmically against the number of moles of water in the incubation mixture divided by the number of moles of rubratoxin B added (mol H_2O/mol rubratoxin B), a curve resulted ($r = 0{\cdot}85$) with a slope of $2{\cdot}098 \times 10^{-4}$ (Fig. 7).

The ip LD_{50} values of rubratoxin B and the carboxylic acid derivative in mice were calculated according to the Miller and Tainter modification of the method of Litchfield & Wilcoxon (1949). As shown in Fig. 8, the LD_{50} of the carboxylic acid derivative was markedly the greater of the two 24 hr after dosing, but by 48 hr the two LD_{50} values had converged to the point of overlapping standard errors.

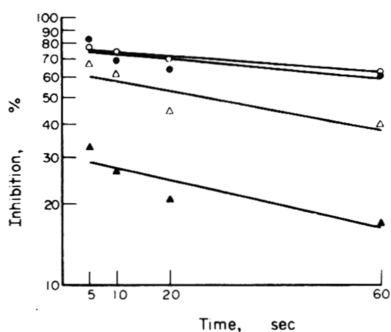

Fig. 6. Semi-log plot of percentage inhibition of Na^+–K^+ ATPase activity by rubratoxin B in reaction mixtures containing $0{\cdot}463 \times 10^3$ (○), $0{\cdot}926 \times 10^3$ (●), $1{\cdot}852 \times 10^3$ (△) or $5{\cdot}555 \times 10^3$ (▲) mol H_2O/mol rubratoxin B after incubation in the mixture for 5–60 sec prior to addition of enzyme. Values represent the means of triplicate assays, with $r = 0{\cdot}962$ (○), $0{\cdot}722$ (●), $0{\cdot}847$ (△) and $0{\cdot}899$ (▲).

DISCUSSION

The decrease in inhibition of Na^+–K^+ ATPase activity with exposure of rubratoxin B to the aqueous incubation mixture prior to addition of enzyme (Fig. 6) was probably the result of rapid conversion

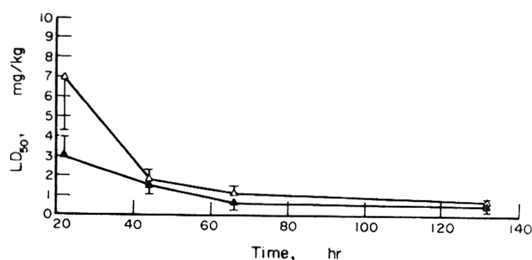

Fig. 8. LD_{50} values (means ± SEM) of rubratoxin B (▲) and its carboxylic acid derivatives (△) calculated 24–132 hr after dosing.

of the toxin to its carboxylic acid derivative (Fig. 4), which has a much weaker inhibitory effect on the enzyme (Table 1). Comparison of the curves in Figs 5 and 7 indicates that the rates of reaction for both conversion of rubratoxin B upon exposure to water and loss of its capacity to inhibit Na^+-K^+ ATPase in aqueous reaction mixtures were exponentially related to the relative abundance of water molecules.

The data in Table 1 indicate either that the carboxylic acid derivative of rubratoxin B has some affinity for Na^+-K^+ ATPase (resulting in a degree of inhibition approximately one-third of that of the parent compound) or that upon addition of the carboxylic acid derivative to the incubation mixture, equilibration with the ring form occurs, resulting in conversion of the carboxylic acid derivative back to the parent form, which is then capable of inhibiting the enzyme. The fact that formation of the carboxylic acid derivative has been shown to be a reversible reaction (Unger & Hayes, 1978) tends to support the latter view. Furthermore, the gross alteration in steric configuration caused by the opening of the anhydride rings makes it unlikely that the two forms would react with the enzyme in the same way.

The toxicity data presented in Fig. 8 also tend to support a slow equilibration between the two forms in the plasma of the whole animal, resulting in a convergence of LD_{50} values by 48 hr. Since the carboxylic acid form is far more polar than the parent form, it is likely to be more rapidly excreted in the urine because it will be less readily reabsorbed in the renal tubules. Moreover, having a much lower lipid–water partition coefficient than the parent compound, the carboxylic acid derivative would be less free to cross the cell membrane to express its toxic potential. Rose & Moss (1970) have shown that opening the maleic anhydride rings of rubratoxin B by Na^+-salt formation resulted in a quadrupling of the ip LD_{50} for mice. The decrease in toxicity of the carboxylic acid derivative seen 24 hr after dosing in the present study was therefore probably due both to the removal of toxic functional groups in the conversion and to the fact that the derivative could cross the lipid barrier of cell membranes less readily than the parent toxin.

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INHIBITORY EFFECT OF CAPSAICIN ON INTESTINAL GLUCOSE ABSORPTION *IN VITRO*

Y. MONSEREENUSORN and T. GLINSUKON

Department of Physiology, Faculty of Science, Mahidol University, Rama VI Rd, Bangkok 4, Thailand

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Abstract—The effects of capsaicin and a crude extract of Capsicum on intestinal glucose absorption have been studied *in vitro*. Glucose absorption in the rat jejunum was slightly stimulated by capsaicin in a concentration of 7·0 mg/100 ml but was inhibited by concentrations of 14·0 or 21·0 mg/100 ml, in 30-min incubations. Similar inhibitory effects on glucose absorption were demonstrated in the hamster jejunum and in intestinal preparations from either species treated with a crude extract of Capsicum in Krebs-Henseleit-HCO_3^- buffer. However, addition of ATP (100 μM) to the mucosal side of a capsaicin-treated preparation restored the level of glucose absorption to the control value. A reduction in the ATP content of the intestinal mucosa observed in the capsaicin-treated jejunum of both rats (by 17·1%) and hamsters (by 36·3%) offers a possible explanation for the inhibitory effect of capsaicin on glucose absorption. The results also suggest that higher concentrations of capsaicin may reduce the ATP content of the intestinal mucosa and thus inhibit intestinal glucose transport, partly by inhibiting the production of mitochondrial ATP.

INTRODUCTION

Capsicum is an important spice which is consumed in many parts of the world. It is one of the main components of curry and is believed to be one of many factors causing gastro-intestinal disturbances (McDonald, Anderson & Hashimoto, 1967). It has been shown to increase acidity, blood flow, DNA content and exfoliation in the stomach (Desai, Venugopalan & Antia, 1973; Ketusinh, Dhorraninta & Juengjareon, 1966; Kolatat & Chungcharoen, 1972). Its main component, capsaicin, plays an important role in its physiological and pharmacological effects on the sensory and cardiovascular systems (Toda, Usui, Nishono & Fujiwara, 1972; Toh, Lee & Kiang, 1955; Webb-Peppole, Brender & Shepherd, 1972). Lee (1963) studied some pathological changes in various organs of rabbits fed high dietary levels of red pepper in long-term experiments and showed that high-fat diets supplemented with red pepper increased the toxicity of capsaicin. More recently, Nopanitaya (1973) reported that rats fed a low-protein diet containing capsaicin showed very poor growth and fat absorption, findings that correlated well with the pathological changes observed in the absorptive cells of the duodenum (Nopanitaya, 1974; Nopanitaya & Nye, 1974).

On the basis of the scanty data available for the effect of capsaicin on the absorption of nutrients, the study described here was undertaken to investigate the abnormalities of intestinal glucose absorption that may be directly related to the growth of experimental animals and man.

EXPERIMENTAL

Materials. Synthetic capsaicin (8-methyl-*N*-vanillyl-6-nonenamide; mol wt 305·4) was obtained from Sigma Chemical Co., St. Louis, MO. A crude extract of Capsicum was prepared by boiling a suspension of 10 g ground red pepper (*Capsicum minimum* L.) in 100 ml Krebs–Henseleit-HCO_3^- buffer for 5 min. After boiling, the solution was immediately filtered through paper and the filtrate was cooled to room temperature. Glucostat reagents were purchased from Worthington Biochemical Corp., Freehold, NJ. All other chemicals were reagent grade.

Animals. Adult male Fischer-derived albino rats (250–275 g) and male Syrian golden hamsters (100–120 g), obtained from the Animal Production Center of the Faculty of Science, Mahidol University, were caged in groups of five and given rat pellets, until the time of the experiment, and water *ad lib.*

Physiological and biochemical methods. The animals were stunned and decapitated, and a 12-cm segment of upper jejunum, beginning from the ligament of Treitz (suspensory muscle of the duodenum), was excised and removed. The jejunum was gently flushed with 0·9% saline and everted (Wilson & Wiseman, 1954). Each sac (3 cm) was filled with 0·5 ml 7·5 mM-glucose in Krebs–Henseleit-HCO_3^- buffer (300–310 mOSM) at pH 7·4 and then immersed in 10 ml 7·5 mM-glucose with or without capsaicin or crude Capsicum extract in Krebs–Henseleit-HCO_3^- buffer contained in a 50-ml Erlenmeyer flask. The incubated solution was aerated with O_2–CO_2, 95:5 v/v, and maintained at 37°C in a shaking water-bath for 30 or 60 min. After incubation, the sacs were removed, blotted and weighed before and after removal of the serosal content. The glucose concentrations on both the mucosal and serosal sides were determined by the glucose-oxidase method (Saloman & Johnson, 1959).

Intestinal mucosal ATP was extracted by the method of Dietrich & Friedland (1959). The technique involved denaturation of the intestinal mucosa by placing the whole intestine in ice-cold 3% perchloric acid for 5 min and then blotting. The denatured mucosa was gently scraped off with a cold spatula. The scraped mucosa was then weighed and homogenized in 10 ml 3% perchloric acid, and the homogenate was centrifuged at 3000 rev/min for 10 min. The

supernatant was neutralized with 2 N-KOH and allowed to precipitate for 30 min, the neutralized supernatant being obtained by centrifuging at 3000 rev/min for 10 min. The ATP content was determined by the Firefly luciferase method (Strechler & McElroy, 1957).

RESULTS

The effects of capsaicin on glucose absorption in everted sacs of the rat and hamster jejunum are summarized in Table 1. The data indicate that capsaicin inhibits the active transport of glucose. Although glucose absorption in the rat jejunum was slightly stimulated by a concentration of 7·0 mg/100 ml and was virtually unaffected by a concentration of 10·5 mg/100 ml, it was significantly inhibited by the presence of higher concentrations, with 14·0 and 21·0 mg capsaicin/100 ml causing reductions of 22·5 and 30·3% respectively. The 14·0 mg/100 ml concentration had an even greater inhibitory effect on glucose absorption in the hamster jejunum, reductions of 29·6 and 37·7% being recorded in 30- and 60-min incubations, respectively.

The inhibitory effect of the crude extract of Capsicum on glucose absorption under similar conditions is shown in Table 2. In the rat jejunum, the degree of inhibition showed little difference between the 30- and 60-min incubations (27·5 and 29·2% respectively) while in the hamster tissue, which was more sensitive than the rat jejunum to the enterotoxic effect of the crude extract, the maximal effect (43·7% inhibition) was recorded after the 30-min incubation.

The relationship between percentage inhibition glucose absorption and the incubation time is shown in Fig. 1 for the rat jejunum. Capsaicin in a concentration of 14·0 mg/100 ml caused some inhibition of glucose absorption in a 15-min incubation, and the subsequent increase with time was linear ($r = 0·9947$), the degree of inhibition after incubation for 30, 45 and 60 min being 20·9, 34·3 and 48·2%, respectively.

Table 3 shows the relationship between glucose absorption and the mucosal content of ATP. Mucosal ATP was 17·1% lower than the control value in rat jejunum after incubation for 60 min in 7·5 mM-glucose with 14·0 mg capsaicin/100 ml in Krebs-Henseleit-HCO_3^- buffer, whereas glucose absorption decreased by 36·1%. The decrease in mucosal ATP was even greater (at 36·3%) in the hamster jejunum, but the difference between the two species in the fall in glucose absorption was much less marked. However, addition of ATP (100 μM) to the buffered 7·5 mM-glucose containing capsaicin at a level of 14·0 mg/100 ml in contact with the mucosal side of the rat jejunum restored the glucose concentration on the serosal side. No further increase in glucose absorption was observed when the concentration of added ATP was doubled. With the restoration of the serosal glucose concentration, following the addition of different amounts of ATP to the mucosal side of capsaicin-treated jejunum, the ATP content in the mucosa also returned to normal levels. In the hamster jejunum, addition of ATP (100 or 200 μM) to the mucosal side of capsaicin-treated hamster jejunum partially restored glucose absorption (to 25·9 and 23·0% inhibition, respectively), while the mucosal ATP was increased to levels of 10·4 and 35·1% of the control figure, respectively.

DISCUSSION

The results presented here clearly show that intestinal glucose absorption is inhibited capsaicin at and above concentrations of 14·0 mg/100 ml in both the rat and hamster. The extent of the inhibition depends upon the capsaicin concentration and the incubation time, the relationship between the latter factor and inhibition of glucose absorption being linear. These findings on the inhibition of glucose absorption are in good agreement with the results of our *in vivo* study, in which 14·0 mg capsaicin/100 ml inhibited glucose transport from the lumen (Y. Monsereenusorn and T. Glinsukon, unpublished data).

Glucose is known to be actively transported in the intestinal mucosal cells. Matthews & Smyth (1960) and Smyth (1961) suggested that the active transport

Table 1. *Effect of capsaicin on glucose absorption in everted sacs of rat and hamster jejunum incubated in Krebs–Henseleit-HCO_3^- buffer (pH 7·4), containing 7·5 mM-glucose and with or without capsaicin, at 37°C for 30 min.*

Species	Capsaicin concn (mg/100 ml)	No. of animals	Final glucose concn (mg/100 ml)		Inhibition (−) or stimulation (+)‡ (%)
			Serosa†	Mucosa†	
Rat	0	20	187 ± 19	102 ± 3	—
	7·0	10	210 ± 31	116 ± 11	+12·3
	10·5	15	183 ± 12	99 ± 3	−2·1
	14·0	12	145 ± 3	92 ± 5	−22·5*
	21·0	8	131 ± 21	100 ± 5	−30·3**
Hamster	0	4	406 ± 17	90 ± 3	—
	14·0	4	286 ± 4	103 ± 4	−29·6**
	0§	7	621 ± 28	78 ± 6	—
	14·0§	8	387 ± 28	83 ± 4	−37·7**

† Values are means ±SEM.

‡ The degree of inhibition or stimulation is derived from a comparison of the test and control concentrations on the serosal side, significant values (Student's *t* test) being indicated by asterisks: $*P < 0·05$; $**P < 0·005$.

§ Incubation for 60 min.

Table 2. *Glucose absorption in everted sacs of rat and hamster jejunum, incubated at 37°C in Krebs–Henseleit-HCO_3^- buffer (pH 7·4) containing 7·5 mM-glucose and with or without crude Capsicum extract*

Species	Crude extract† (ml)	No. of animals	Incubation time (min)	Final glucose concn (mg/100 ml) Serosa‡	Mucosa‡	Inhibition§ (%)
Rat	0	10	30	189 ± 5	105 ± 4	—
	10	10	30	137 ± 8	123 ± 14	27·5*
	0	8	60	240 ± 20	80 ± 5	—
	10	8	60	170 ± 11	83 ± 3	29·2***
Hamster	0	4	30	415 ± 7	92 ± 3	—
	10	4	30	228 ± 8	120 ± 2	43·7**
	0	10	60	610 ± 22	77 ± 6	—
	10	10	60	412 ± 30	114 ± 8	32·5***

† The crude extract contained 5·26 mg capsaicin/100 ml, as determined by the method of Karawya, Bulbaa, Girgis & Youssef (1967).
‡ Values are means ±SEM.
§ The degree of inhibition is derived from a comparison of the test and control concentrations on the serosal side, significant values (Student's *t* test) being indicated by asterisks: *$P < 0·05$; **$P < 0·005$; ***$P < 0·001$.

of glucose across intestinal mucosal cells takes place in two stages. In stage I, phlorhizin abolishes the mucosal transport at a concentration that still permits considerable metabolism of glucose derived from the serosal fluid (Parsons, Smyth & Taylor, 1958). In contrast, dinitrophenol and anoxia abolish glucose transport from the serosal side, while still permitting the entry of glucose into the mucosal cells from the mucosal side, in what is known as stage II. Thus, stage II of the active transport of glucose is dependent upon metabolic energy. According to this hypothesis, capsaicin may be classified as a second-stage inhibitor, since it decreases the mucosal ATP content, with consequent inhibition of intestinal glucose transport, and addition of 100 μM-ATP to the mucosal side significantly reverses the capsaicin-depression of the glucose concentration on the serosal side. The fact that no greater restoration of this glucose concentration was brought about by addition of 200 μM-ATP is probably due to an inability of the intestinal mucosal cells to increase their uptake of exogenous ATP with increasing concentration above a maximum level of 100 μM (Chaudry, Sayeed & Baue, 1976) and to the spontaneous degradation of some exogenous ATP in *in vitro* rat-intestinal preparations (Bronk & Leese, 1973). The inhibitory effect of capsaicin on glucose absorption may reflect a reduction in mucosal ATP either through an increase in intracellular ATP hydrolysis, leading to impaired cellular function, or through a decrease in ATP liberated from an energy-generating system. The latter is a possible mechanism, since capsaicin has been shown to inhibit ATP production in isolated rat-liver mitochondria (Chudapongse & Janthasoot, 1976). These authors suggest that capsaicin has two sites of action on the energy transfer pathway of mitochondria. One is the phosphorylation mechanism and the other appears to involve certain energy-conserving reactions above the site of action of dinitrophenol. Moreover, since Na^+-K^+ ATPase is known to play a part in the active transport of glucose (Fujita, Ohta, Kawai, Matsui & Nakao, 1972), interference with the activity of this enzyme may alter glucose absorption. In fact, it was found that capsaicin depressed dinitrophenol-activated ATPase activity in isolated rat-liver mitochondria (Chudapongse & Janthasoot, 1976). Therefore, it is suggested that capsaicin may inhibit Na^+-K^+ ATPase activity in the intestinal mucosal cells and that this may in turn reduce glucose absorption. However, the action of capsaicin on Na^+-K^+ ATPase activity in the intestinal mucosal cells is under investigation in this laboratory.

In the hamster jejunum, the inhibitory effect of glucose absorption by capsaicin is quite different from that found in the rat after addition of exogenous ATP. It was found that the mucosal ATP content was markedly increased (to 35% above the control value)

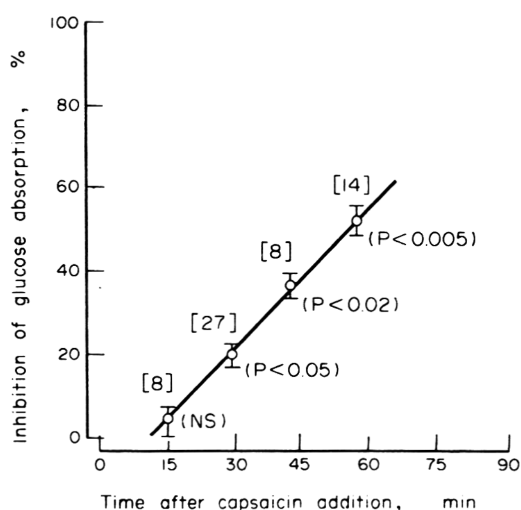

Fig. 1. Relation between incubation time and percentage inhibition of glucose absorption in everted sacs of rat jejunum incubated in 7·5 mM-glucose with or without 14·0 mg capsaicin/100 ml Krebs–Henseleit-HCO_3^- buffer (pH 7·4) at 37°C. Regression analyses were calculated according to Walpole & Myers (1972), the regression line equation being $Y = -13·68 \pm 1·06\,X$. The numbers of animals used are indicated in brackets. NS = Not significant.

Table 3. *Effect of addition of ATP on glucose absorption in everted sacs of rat and hamster jejunum incubated at 37 C in Krebs–Henseleit-HCO$_3$ buffer (pH 7·4) containing 7·5 mM-glucose and with or without 14·0 mg capsaicin/100 ml*

Species	Treatment on mucosal side: Capsaicin (mg/100 ml)	ATP (μM)	No. of animals	Decrease (−) or increase (+) in mucosal ATP† (%)	Final glucose concn (mg/100 ml): Serosa‡	Mucosa‡	Inhibition§ (%)
Rat	0	0	10	—	216 ± 22	79 ± 4	—
	14·0	0	6	−17·1*	138 ± 20	90 ± 8	36·1*
		100	12	−8·4	198 ± 14	76 ± 5	8·3
		200	12	−4·8	203 ± 10	88 ± 5	6·0
Hamster	0	0	8	—	625 ± 28	77 ± 6	—
	14·0	0	8	−36·3***	378 ± 28	83 ± 4	39·5***
		100	11	+10·4	463 ± 14	76 ± 2	25·9**
		200	11	+35·1*	481 ± 36	80 ± 2	23·0**

† Percentage change in mucosal ATP was calculated from control values of 1·60 ± 0·18 and 1·46 ± 0·14 μmol/g wet weight of tissue for the rat and hamster, respectively.
‡ Values are means ±SEM.
§ The degree of inhibition is derived from a comparison of the test and control concentrations on the serosal side. Significant values (Student's *t* test) are indicated by asterisks: *$P < 0·05$; **$P < 0·01$; ***$P < 0·005$.

but the glucose concentration on the serosal side was not even restored to the control value. This finding indicates a probable decrease in the ability of the intestinal mucosal cells to hydrolyse intracellular ATP. Capsaicin probably inhibits the Na^+–K^+ ATPase activity and in turn leaves an increased mucosal ATP content. Furthermore, Na^+–K^+ ATPase activity was significantly decreased in the jejunal mucosa treated *in vitro* with 14·0 mg capsaicin/100 ml (Y. Monsereenusorn and T. Glinsukon, unpublished data). This supports the supposition that capsaicin inhibits Na^+–K^+ ATPase activity.

A similar inhibitory effect on glucose absorption was observed with the crude extract of Capsicum, although the extent of the response differed with incubation time and the species of animal used. However, this inhibition of glucose absorption was effected with crude extract containing capsaicin in a concentration of only 5·26 mg/100 ml (Table 2). At this concentration, pure capsaicin would not inhibit glucose absorption and might even bring about a slight stimulation, as was shown with the concentration of 7·0 mg pure capsaicin/100 ml. These findings suggest that other compounds present in the crude extract may reinforce the enterotoxicity of capsaicin.

The results presented in this study thus indicate that capsaicin, a pungent agent of Capsicum, inhibits glucose absorption when it is in direct contact with mammalian small intestine *in vitro*. It is not yet known whether a similar inhibition may occur in man when capsaicin is ingested. However, should there be such an inhibitory effect on the active transport of glucose or possibly of amino acids, the consequences are likely to be significant, especially in people who are on a marginal diet.

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SHORT PAPER

EFFECTS OF LOW LEVELS OF BUTYLATED HYDROXYTOLUENE ON THE PROTHROMBIN INDEX OF MALE RATS

O. TAKAHASHI and K. HIRAGA

Department of Toxicology, Tokyo Metropolitan Research Laboratory of Public Health, Tokyo 160, Japan

(*Received 12 April 1978*)

Summary—Butylated hydroxytoluene (BHT) was administered in the diet to groups of five male rats at concentrations of 0·0085, 0·017, 0·033, 0·065, 0·13, 0·25 and 0·50% for 1 or 4 wk. A significant decrease in the prothrombin index was observed at wk 1 in each group of rats fed BHT levels of 0·017% or more and this effect was still apparent at wk 4 in the group given 0·50% BHT.

Introduction

Butylated hydroxytoluene (BHT) is a hindered phenolic antioxidant widely used in food products and in rubber and petroleum chemicals. We have reported the induction of haemorrhagic death in rats by dietary BHT (Takahashi & Hiraga, 1978), and although the detailed mechanism of this phenomenon is unknown, hypoprothrombinaemia appeared to be an important factor in this haemorrhage.

We therefore attempted to study the prothrombin-decreasing effect of low levels of BHT and to evaluate the safety of BHT as a food antioxidant in relation to its haemorrhagic effects.

Experimental

Male Sprague–Dawley rats, obtained when 4 wk old from CLEA, Tokyo, were fed a laboratory ration (CLEA CE-2) for 2 wk and then caged in groups of five in a conditioned room at 25 ± 1°C and 50 ± 5% relative humidity. These groups were fed either powdered CLEA CE-2 alone or this basal diet containing BHT (from Tokyo Kasei Kogyo Co.) at a level of 0·0085, 0·017, 0·033, 0·065, 0·13, 0·25 or 0·50% for 1 or 4 wk. The composition of the diet and other details of procedure were the same as those described by Takahashi & Hiraga (1978).

During the experimental period, food consumption and body weights were recorded and BHT intakes were calculated. Prothrombin time was estimated at the end of each feeding period by the one-stage method of Quick, Stanley-Brown & Bancroft (1935) and terminal liver weights were determined. The prothrombin index was derived by a previously described formula (Takahashi & Hiraga, 1978).

Results

Initial and terminal body weights and the growth rates for the eight groups are shown in Table 1. The mean daily intakes of BHT, calculated from food intakes and body weights recorded on day 4 for the groups fed for 1 wk and on day 19 for those fed for 4 wk, are also shown in Table 1.

After 1 wk each group of rats on a dietary level of BHT equal to or exceeding 0·017% had a significantly decreased prothrombin index, while at 4 wk only the group consuming 0·50% BHT showed a decrease in this index (Table 2). A significant increase in relative liver weight was observed in the groups receiving 0·13 or 0·50% BHT for 1 wk and 0·50% BHT for 4 wk (Table 2). The fact that the difference between the mean liver weight for the controls and for the group treated with the intervening dose of BHT (0·25%) lacked statistical significance may be attributable to the wide spread of liver weights in the five rats of the latter group; an inexplicably low weight in one animal led to a standard deviation of 0·80 for this group compared with 0·084 for the control group.

Discussion

Hypoprothrombinaemia, which appeared to play a central role in the fatal haemorrhage induced by BHT in an earlier study in rats (Takahashi & Hiraga, 1978), was observed at the end of wk 1 even with a mean level of ingestion of only 14·7 mg BHT/kg/day, but the effect was transient and not haemorrhagic. The haemorrhagic deaths previously reported occurred after BHT feeding had been in progress for 10 days or more, depending on the dose (Takahashi & Hiraga, 1978); therefore, a persistent decrease in the prothrombin index of rats given a daily dose above about 200 mg/kg suggests that this level of BHT is the minimum dose for induction of haemorrhage after an appropriate latent period.

The effects of BHT on liver weight have been reported by many authors and the enlargement of the liver has been described as a physiological change associated with an increase in drug-metabolizing enzymes (Botham, Conning, Hayes, Litchfield & McElligott, 1970; Gaunt, Feuer, Fairweather & Gil-

Table 1. *Mean body weights and food intake of rats fed diets containing 0·0085–0·50% BHT for 1 or 4 wk*

Dietary level (%)	Body weight (g) Initial	Body weight (g) Final	Body-weight gain (%)	Mean food intake (g/rat/day)	Mean BHT intake (mg/kg/day)
			Wk 1		
0	212	257	21	25·9	—
0·0085	216	269	25	21·8	7·54
0·017	220	272	24	21·7	14·7
0·033	223	272	23	26·0	34·1
0·065	223	272	22	24·2	62·5
0·13	224	269	20	24·7	129
0·25	226	270	19	22·4	227
0·50	228	263	15	25·9	529
			Wk 4		
0	217	373	72	28·6	—
0·0085	218	392	80	27·5	6·73
0.017	220	394	79	27·1	13·4
0·033	221	392	78	31·5	29·9
0·065	221	390	76	28·3	52·9
0·13	223	380	71	25·0	96·0
0·25	224	369	65	31·0	230
0·50	224	375	67	21·8	326

Values are means for groups of five rats.

bert, 1965; Gilbert & Golberg, 1965). The decrease in the prothrombin index was a more sensitive response than liver enlargement, but like the increase in liver weight it had returned to normal by wk 4 in animals given dietary levels of BHT below 0·50%. There seem to be two possible explanations for this sequence of events. Either the decrease in the prothrombin index of rats given low levels of BHT is an undefined adaptive change related to the induction of hepatic microsomal enzymes or it may depend on the production of metabolites that induce hypoprothrombinaemia, the concentrations of such metabolites being higher during wk 1, because of a low level of induction of the detoxicating enzymes, and subsequently being decreased by rapid detoxication associated with adaptive changes in hepatic function. In any event, hypoprothrombinaemia must be considered a toxic response because there is no evidence to support a teleological explanation, even if such an explanation is accepted for the induction of drug-metabolizing enzymes. Although haemorrhage induced by BHT is prevented with dietary supplements of vitamin K and the safety of BHT in relation to haemorrhage cannot be evaluated on the basis of data only from rat experiments, because of the species difference in this BHT effect (O. Takahashi, unpublished data 1977), it is notable that the minimum daily intake that caused significant hypoprothrombinaemia

Table 2. *Mean prothrombin index and relative liver weight of rats fed diets containing 0·0085–0·50% BHT for 1 or 4 wk*

Dietary level (%)	Values after 1 wk: Prothrombin index (%)	Values after 1 wk: Relative liver weight (g/100 g body weight)	Values after 4 wk: Prothrombin index (%)	Values after 4 wk: Relative liver weight (g/100 g body weight)
0	100	6·07	100	4·94
0·0085	90·5	6·12	97·9	4·68
0·017	85·9*	5·92	100	5·15
0·033	82·2*	6·23	98·4	5·35
0·065	78·4**	6·15	95·9	5·08
0·13	80·9**	6·69***	105·6	5·25
0·25	84·4*	6·89	81·4	5·04
0·50	73·8**	7·46***	78·9**	6·21***

All values are means for groups of five rats and those marked with asterisks differ significantly (Student's t test) from the appropriate control values: $^{*}P < 0·05$; $^{**}P < 0·01$; $^{***}P < 0·001$.

at wk 1 was about 14·7 mg/kg, a dose less than 100 times the acceptable daily intake for man recommended by the Joint FAO/WHO Expert Committee on Food Additives (1976).

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Review Section

TOXICOLOGY STUDIES. I. SUPPORT PERSONNEL

D. L. ARNOLD,
Bureau of Chemical Safety, Food Directorate, Health Protection Branch, Ottawa, Canada

J. G. FOX,
Division of Laboratory Animal Medicine, Massachusetts Institute of Technology, Cambridge, MA, USA

P. THIBERT
Animal Resources Division, Health Protection Branch,

and

H. C. GRICE
Bureau of Chemical Safety, Food Directorate, Health Protection Branch, Ottawa, Canada

(Received 3 April 1978)

Summary—The successful execution of any toxicological programme is mainly dependent upon the competence and diligence of the technical staff working with the animals. A special aptitude for handling and a willingness to care for animals is an inherent trait of these individuals. Their educational background and training are more sophisticated and their experience and responsibilities should be more exacting today than ever before, since technological advances have allowed toxicological research to monitor changes at the molecular level. New management procedures should be adopted to attract and retain such highly trained and motivated individuals.

Introduction

At the present time, legislation in the United States and Canada imposes on manufacturers the responsibility for demonstrating satisfactorily that a chemical is 'safe' and efficacious for its intended use. To comply with this legislation, a toxicity testing programme is initiated under the direction of the manufacturer. Suitable guidelines exist for laboratories conducting toxicological research programmes (Food and Drug Administration, 1971; Friedman, 1970; Hagan, 1959; Health and Welfare Canada, 1975; Shubik, 1970; Sontag, Page & Saffiotti, 1976), and it had been assumed that all laboratories were using appropriate experimental procedures in their toxicity testing programmes, but the US Food and Drug Administration (FDA) has recently questioned the manner in which some laboratories have conducted their toxicity tests (*Federal Register*, 1976). The FDA and other US governmental agencies have therefore proposed very detailed standard operating procedures for laboratories conducting toxicological testing. The proposed legislation encompasses Good Laboratory Practices. Whether this legislation will recognize the inherent differences between the types of toxicological tests conducted in academic, contract, and research and development laboratories remains to be seen.

Since many practices in the design and conduct of various toxicity tests are poorly defined, it is our intention to consider this problem, paying particular attention to the logistics, management and control of a toxicological research or safety assessment programme. This paper, the first of a series, will consider the support staff. Subsequent papers will be concerned with such topics as the ways of differentiating between disease and toxic response, conducting a clinical examination and using clinical tests as diagnostic aids and the means by which the toxicologist obtains a 'healthy' animal.

Prior to the 1970s, monitoring animals during a toxicity test consisted essentially of obtaining data on body weight and feed consumption and assessing, in a very superficial manner, the general health of the animal. The introduction of training in laboratory animal science and medicine, and the recognition of the particular dietary and housing requirements of laboratory animals has produced a healthier research animal and possibly more valid results than were previously attainable. This, in conjunction with the technological developments that have resulted in common usage of such investigational tools as radioisotopes, sensitive and accurate chromatographic procedures and the electron microscope, has greatly extended the toxicologist's ability to ascertain changes at the molecular level. Toxicological research now requires a multidisciplinary team approach. The minimal core team designing such experiments includes the toxicologist, pathologist and statistician and a laboratory animal specialist. Depending upon the complexity of the project and what is known about the test material, a biochemist, epidemiologist, clinical chemist, analytical chemist, kineticist, haematologist, nutritionist, microbiologist and/or computer programmer may contribute to a toxicological study. An analytical laboratory is also an inherent part of any toxicity test, to ascertain the purity of the test compound and

its stability both during storage and when mixed with the vehicle (food, water or solvent) for administration.

The quality of any toxicology programme undertaken by a multidisciplinary team is dependent upon the weakest component of the team. Use of sophisticated technical and analytical equipment for monitoring various biological and chemical parameters has increased the possibility of detecting subtle effects. However, emphasis on technology of this kind has, at times, been at the expense of provisions for the maintenance of healthy animals. Successful maintenance and monitoring of animal health is dependent upon the competence and diligence of the technical staff working with the animals. The longer the study, the greater is the requirement for a competent staff. Although this article will discuss various aspects of matters concerning the technical support staff, their formal education, practical experience, training requirements, responsibilities and management, the prime emphasis will be upon the technical staff working with the animals. The following considerations are based primarily on a programme that has evolved over a number of years and has been successful in our laboratories. While other laboratories may have different, but still satisfactory, programmes, the basic concepts outlined here should be applicable to a variety of programmes of toxicological research or safety testing.

Animal technicians

The knowledge required of the animal technician in any toxicity testing programme depends upon the type of research undertaken. For the purpose of this discussion, the animal technicians will be classified according to the American and Canadian Associations for Laboratory Animal Science (AALAS and CALAS, respectively), but the specific qualifications and duties, excluding certification requirements, for each grade of animal technician are those assigned by the authors.

The Laboratory Animal Technician (LAT) is primarily responsible for the husbandry aspects of animal care. The Senior Laboratory Animal Technician (SLAT) is involved with the day-to-day care, handling and health monitoring of the animal colony. The Master Laboratory Animal Technician (MLAT) supervises the other technicians, and consults daily with the project scientist and regularly with the veterinarian in charge of the animal colony. In effect, the MLAT monitors the day-to-day progress of the assigned experiments.

The essential attributes for any animal technician include a special aptitude for handling and a willingness to care for animals. Such individuals are necessary for the maintenance of the desired standards of husbandry within an animal colony. The job must be sufficiently attractive to the technician to ensure a minimal turnover of personnel. Attainment of such a goal requires that a career development programme be available to help ensure that the job is both stimulating and rewarding (Holmes & Smith, 1977; Neil & Layne, 1976).

Laboratory Animal Technician (LAT)

The minimal educational requirements for this position in most toxicology or safety evaluation laboratories is a high-school diploma. Some facilities have less stringent requirements but encourage the attainment of a high-school diploma while the technician is on the job.

The LAT is required to perform duties concerned with the general care, breeding and maintenance of a wide variety of laboratory animals, and will undergo a training programme, which includes:

(i) the handling and/or restraining of various laboratory animal species;
(ii) instruction in the principle, practice and responsibilities of a barrier-maintained animal colony;
(iii) general training concerning the nutritional requirement of various species;
(iv) the proper cleaning and/or sanitizing of the animal rooms, cages, water bottles and related facilities, following established procedures and schedules;
(v) the operation of equipment used to sanitize animal cages and water bottles;
(vi) the following of safety precautions during the performance of the above responsibilities.

Some personnel will carry out the above duties in a satisfactory manner but do not desire any greater job responsibility or further training. Other individuals are more concerned with career development and willingly undertake additional training with the hope of attaining a more challenging position. However, most technicians find this job boring and may become careless in carrying out their duties. One management practice utilized to reduce the incidence of boredom, is to assign in an arbitrary way approximately 75% of the LAT's time for the above duties, the remaining time being spent on improving the LAT's technical skills through an internal training programme.

Certification of a LAT by AALAS or CALAS requires a year's experience as a full-time employee in an animal facility providing the above training.

Senior Laboratory Technician (SLAT)

The minimal educational prerequisite for this grade is a high-school diploma. Certification as a SLAT requires that an individual holds a LAT certification and has at least 3 years experience as a full-time animal technician in an animal facility offering approved experience in animal care and use. Such individuals are often required to undergo training during off-duty hours. Both AALAS* and CALAS† have educational guidance packages available for animal-care personnel. The packages contain training manuals, slides, cassettes, specific references to literature and additional applied hand-out material that is suitable and contemporary. Additionally, many colleges and technical institutions now offer courses in Laboratory Animal Care.

The responsibilities of the SLAT include:

(i) monitoring the animals health on a daily basis

*AALAS National Office, 2317 West Jefferson St., Suite no. 208, Joliet, IL 60435.

† CALAS National Office, 2627 Morley Trail, N.W. Calgary, Alberta T2M 4G6.

and performing perfunctory clinical examinations;
(ii) recording the body-weight and feed-consumption data and ascertaining that the correct diet is fed;
(iii) keeping a daily log book for recording environmental variables (temperature and humidity) and observations concerning any animals that require additional health monitoring;
(iv) identification of each animal with a unique and permanently attached code, such as a tattoo, ear tag, toe slip, ear punch or neck chain (see New, 1977) and the maintenance of appropriate records;
(v) special duties, which may include administration of test chemicals, examination for parasite ova, collection of blood, urine or tissue samples, use of metabolic cages, diagnosing pregnancy, and assisting with autopsies and the preparation of quality-controlled diets according to precise formulations.

The in-house training of the SLAT is primarily under the supervision of the Master Laboratory Animal Technician, but occasionally it may involve the use of appropriate articles from the scientific literature, lectures on work-related subjects and possibly special instructions concerning data-gathering for electronic processing.

Master Laboratory Animal Technician (MLAT)

Many institutes require this individual to have a Bachelor of Science degree in Biology or to have completed successfully a 2- or 3-year course in animal care and technology at a community college or technical institute. For CALAS or AALAS certification, this individual must have a SLAT certificate, with approximately 3–6 years of full-time employment in laboratory animal technology, depending upon education. The candidate for certification must have a comprehensive and detailed knowledge of laboratory animal care, facilities, equipment design, administration and the experimental use of laboratory animals.

The responsibilities of the MLAT include:
(i) supervising technicians, determining their work loads and overseeing the quality of their work;
(ii) assisting in the development of experimental protocols with the project scientist;
(iii) monitoring the health of the test animals, tentatively diagnosing disease conditions and consulting with the veterinarian about diseased animals;
(iv) conducting detailed clinical examinations and assisting the pathologist with autopsies;
(v) co-ordinating the logistics necessary for the pathological, clinical chemistry and biochemical requirements during a study;
(vi) providing part of the in-house training for the LATs and SLATs;
(vii) having the ability to 'trouble-shoot' laboratory equipment and to obtain the necessary assistance for its repair;
(viii) assisting in the preparation of progress or final reports and verifying the data.

Depending upon the requirements of the research facility, a training programme for MLATs could be structured as follows:
(i) Lecture programmes: Lectures and instruction in gross and microscopic anatomy, physiology, nutrition, clinical chemistry and basic laboratory pathology of the common laboratory species may be offered on a continuing schedule. In our programme, particular attention is given in these sessions to the disease conditions commonly encountered in our laboratory. Examples of the type of lecture subjects covered include consideration of inflammatory and degenerative lesions, the growth and distinguishing characteristics of a tumour, the common laboratory parasites and their control, and basic considerations of bacterial and virus diseases. Additional lectures are concerned with the animal's environment, particularly the micro-environment, and with the ways in which alterations in the environment can influence an animal physiologically, leading to changes in response to various microbial and test chemicals (Fox, 1978). Occasionally, a specific refresher course may be required, for example, on the preparation of solutions when a study entails the frequent preparation of dosing solutions or reagents.
(ii) Reading and technical seminars: These consist of assigned reading of selected chapters from books and journals on laboratory animal care and disease, toxicology and pathology. A sample of such books is given in the appended bibliography. The reading assignments are co-ordinated with periodic technically-orientated seminars in which senior technicians are expected to participate either as speakers or discussants.
(iii) Special instruction: This training involves the use of basic or special clinical diagnostic equipment such as the stethoscope, ophthalmoscope, slit lamp, sphygmomanometer and thermometer. The MLAT also becomes familiar with diagnostic procedures that require manipulation of the animal to detect visceral or subcutaneous tumours.

In view of the amount of data that can be generated during a toxicity study even as short as 90 days, it is almost imperative that some type of electronic data processing be used. The MLAT is the person primarily responsible for gathering and authenticating the data, and consequently requires a basic understanding of a computer system. In addition, the MLAT may have to serve as a back-up operator when data must be processed within a 24-hr period, as when body-weight and food-consumption data serve as a component in the overall clinical assessment of the animal (Arnold, Charbonneau, Zawidzka & Grice, 1978).

Some other managerial considerations are applicable to all animal technicians:
(i) To minimize the possibility of spreading disease throughout an animal colony, animal technicians should be assigned to a minimum number of studies. Moreover, in order to avoid undue stress to the animals, it is preferable that animal technicians be assigned to a study for its duration or for a significant part of it, so that the animals become accustomed to one individual. The technician also becomes familiar with

the animals' behavioural patterns and therefore easily recognizes changes that could signify impairment of an animal's health. However, this may be an impractical procedure for monkeys, who occasionally become so 'accustomed' to one technician that they become completely unresponsive to another individual (J. Truelove, personal communication 1977).

(ii) Prior to the starting of a study, it is imperative that all the animal technicians fully understand its objective and significance and their responsibilities and role as a member of the team. In addition, expected behavioural or clinical changes should be discussed. The technicians must be cognizant of safety precautions, for example, when handling radioisotopes or carcinogenic chemicals. A technician may not fully appreciate the need to use protective equipment or clothing for such routine tasks as disposal of contaminated bedding materials, which may not constitute an immediately recognizable health hazard.

(iii) Any study lasting several months will be affected by vacation schedules and sick leave. Therefore, sufficient support staff must be available to cover these eventualities. This is efficiently accomplished if the temporary replacement has prior knowledge of the on-going study and requires only a brief orientation to fulfil the expected duties.

(iv) During any long-term study, the loss of animals before pathological examination as a result of autolysis can be reduced drastically with a diligent health-monitoring programme (Arnold *et al.* 1978). Some research facilities require a pathologist to be on call during normal off-duty hours, but it has been our policy to train every animal technician to prepare animals for a subsequent autopsy. They are taught the proper procedures of killing, evisceration, brain and pituitary gland excision, and storage of tissues in suitable fixatives for examination by a pathologist.

Veterinary service personnel

The veterinarian ensures that only healthy and suitable animals are used for experimentation. He/she diagnoses the disease of laboratory animals, performs or supervises clinical tests, prescribes medication, details the dosing schedule, dispenses pharmaceuticals and provides veterinary surveillance of all research activities within the animal colony. The veterinarian assists in the development of new animal models for research purposes and participates in the management of animal care.

Veterinary technicians, who have received advanced training in veterinary technical services at various Agricultural Colleges in the USA or Colleges of Technology in Canada, assist the veterinarian. They have received training in histology, microbiology, haematology, bacteriology, clinical chemistry and animal husbandry and are responsible for the daily treatment, care and surveillance of an animal after the veterinarian has given his diagnosis and prescribed a treatment schedule. These individuals can provide routine surgical services such as the installation of indwelling catheters or cannulation of bile ducts.

The need for an individual with this type of training often depends upon the size of the animal colony and type of research programme.

Organizational considerations

Facility usage

A toxicologist's requirement for animal housing, husbandry personnel, special diets and other facilities is usually of a cyclic nature. With the increasing cost of labour, equipment and facilities, many institutions have centralized all of the animal care facilities under the control of one individual, designated manager of the Animal Care Unit, in order to utilize resources more efficiently. By co-ordinating all pending research programmes through this manager, the Animal Care Unit can, in theory, provide a tailor-made service for every researcher's requirements. Consequently, this individual must have appropriate managerial experience to function effectively and efficiently, if the desired objectives are to be attained.

Career development

Some type of career development programme is required if highly trained and motivated individuals are to become animal technicians. The implementation of a career development programme within an Animal Care Unit requires a great deal of time and thought. Although a detailed consideration of this question is beyond the scope of this paper, some of the basic components of such a system include:

(i) the establishment of grades or levels of animal technicians, with defined requirements relating to education and experience for each grade;

(ii) a definitive procedure to be followed and specific requirements for an individual to progress within and between grades;

(iii) a means of establishing that an individual aspiring to achieve the next grade can perform specific functions in a satisfactory manner and a mechanism for an individual to progress within a grade prior to the achievement of the next grade;

(iv) some type of competition for filling a senior position that becomes vacant, since the more senior grades may require that an individual occupies a specific job;

(v) some arrangement whereby an individual's willingness to undergo extramural job-related training could be partially subsidized and be accorded some extra weight in an application for an advancement.

Other technical personnel

The types of technical expertise that may be required in a major toxicological testing programme include histology, haematology, clinical chemistry, microbiology, analytical or organic chemistry and biochemistry. Their compilation under one heading is not an attempt to diminish their contribution to any toxicological evaluation, but since the requirement for such services will vary between studies, there may not be sufficient justification to have such individuals on the permanent staff. In most instances, con-

tract laboratories are available to fulfil such requirements and to provide expertise on an 'as needed' basis.

Types of records for a toxicity study

Log books

A daily log book is kept in each animal room, for recording the following types of information:

(i) Temperature and humidity: Although it is ideal to record such information with sensitive equipment on a continuous 24-hour basis, a minimum of two observations a day should be made at approximately the same times each day. Such data may provide an explanation for a sudden change in behaviour or in food and water consumption.

(ii) Equipment maintenance log: A scheduled list is provided for preventive maintenance and the procedure to be followed in any case of equipment malfunction. A description of all equipment malfunctions, the corrective action taken and the length of time the equipment was not operative are entered in the daily log book. Any changes that are observed in the test animals and can be attributed to environmental change resulting from equipment malfunction are also noted.

(iii) Clinical observations: Any deviation from the normal behavioural pattern is recorded (both in the log book and on the clinical sheet) and a note is made that the animal requires additional monitoring.

(iv) Mortality: The identification number of any animal dying during the day and its disposition is recorded.

(v) Intensive Care Unit (ICU): One aspect of health monitoring during a chronic feeding study may involve the use of an ICU (Arnold *et al.* 1978). Body weight and feed consumption of animals housed within the ICU are recorded daily in the log book rather than on the clinical sheet.

Clinical sheets

An individual clinical sheet for each animal is useful during a subacute or chronic feeding study for recording the results of all clinical examinations or organ function tests. The sheet can be used to deduce an approximate latent period when a grossly observed tissue mass is histologically diagnosed as a tumour, to correlate chemical observations with the pathologist's findings at autopsy and to correlate such data with the microscopic evaluation of tissues for a final diagnosis.

The clinical sheet can be quite simple. The heading contains the study number and the animal's identification code, sex and dietary treatment. The remainder of the sheet is blank initially: all entries start with the date, list the observations made and conclude with the initials of the individual making the observation. When an animal dies or is killed, the date, clinical findings, body weight and food consumption are recorded.

Pathology sheets

These contain information concerning the gross observations made at autopsy as well as the microscopic findings.

Body weight/feed consumption data

Periodicity for determining body weight and feed consumption vary from 1 to 4 weeks in a chronic feeding study. These determinations are time-consuming and costly. Of the many computer-based data acquisition and retrieval systems available, the one reported by Munro, Charbonneau & Willes (1972) combines body weight, feed consumption and clinical findings. This information is then used to assess the animal's health status on a weekly basis (Arnold *et al.* 1978). A more complex computer record-keeping system for large rodent colonies has been described by Kalbach, McCallum, Konvicka & Holland (1977). For a discussion concerning the maintenance of research-data integrity inherent in the use of automated information systems see Cranmer, Lawrence, Konvicka, Taylor & Herrick (1976).

Clinical tests

In a subacute study it is common practice to examine various haematological parameters and to use automated equipment to study numerous serum enzymes and elemental components of blood and urine. These findings may help to elucidate the histochemical mechanism of toxicity or to establish the dosage levels for a chronic study. However, since many clinical tests are invasive in nature and constitute a stress on the animal, this may compromise the objective of a chronic study. The judicious use of haematological and clinical chemistry tests is encouraged, provided there is sufficient biological justification, but the availability of sophisticated equipment does not constitute such justification.

Neurological sheets

The neurological assessment of laboratory animals will probably never reach the degree of precision that is possible in man. However, books are available detailing methods for the neurological assessment of some of the larger laboratory animals such as the dog (Hoerlein, 1971; McGrath, 1960). With very little effort, many of these procedures can be adapted for use with rodents. The diligent animal technician with adequate experience can often determine the cause of paralysis, abnormal posture or other nerve-related changes by correlating the neurological assessment with observations at autopsy and histopathological findings.

Conclusions

In summary, the motivation and capabilities of the support personnel have a significant effect upon the success of any toxicological research programme. Although several scientific disciplines are usually involved in any toxicity study, the technicians working directly with the animals have a major responsibility, especially in a chronic study. The educational requirements and experience of such individuals are more exacting today because of advancing technology and the present standards for toxicological testing. An ability to attract, retain and manage such personnel is as important to the toxicologist as the knowledge of the scientific discipline itself. The core team designing any toxicity study should include a laboratory animal specialist, and the efficient utilization of manpower

and resources has encouraged the establishment of an Animal Care Unit to co-ordinate all studies so that personnel and facility requirements can be appropriately tailored for each study. The realization of these objectives cannot be served by describing any one particular programme, but unless it has one already, each institution should consider the implementation of such a programme, tailored to its own prevailing conditions, as a step towards upgrading its toxicological testing.

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BOOK REVIEWS

Trace Substances and Health. A Handbook, Part I. Edited by P. M. Newberne. Marcel Dekker, Inc., New York, 1976. pp. ix + 398. Sw.fr. 120.00.

The scope of this toxicological handbook is broad but not comprehensive, concentrating on enterotoxins, mycotoxins, pesticides, food chemicals and additives, and trace elements. There is no mention of many other important subject areas, such as industrial hazards or cosmetics; indeed the divisions within the book tend to restrict coverage almost entirely to food. Perhaps the gaps will be filled by subsequent parts, since this is Part I, but there are no clues to the possible content of future instalments.

Each of the chapters was contributed by a different expert in the particular field. Chapter 1 on enterotoxin-mediated disease concentrates on six of the most important enterotoxin-producing bacteria and compares the toxicity of these and other bacterial toxins. The toxigenic moulds and the chemical groups of toxins that they produce are given separate treatment in the second chapter. A surprisingly thorough coverage of the vast group of chemical pesticides forms the next chapter. As well as the biological effects of certain pesticides, their interactions and analysis are covered. The final section of this chapter describes the diagnosis and treatment of various forms of pesticide poisoning. Chapter 4 encompasses the whole field of food chemicals and food additives, but detailed coverage is necessarily limited to a few specific compounds, such as monosodium glutamate, brominated vegetable oils, carrageenan and amaranth. The last chapter gives individual treatment to 14 of the more toxic trace elements. The effects of a deficiency of these elements is included, along with the toxic effects of excesses.

Each chapter is thoroughly segmented into sections and subsections, at the expense of readability in places. The numbering of the sections also seems destined to confuse anyone casually delving into these pages. The chapters on mycotoxins and pesticides are well-provided with chemical diagrams but there are few other illustrations in the book. However, reference lists are very thorough, totalling over 1200 citations.

This book will probably be most valuable to the student or non-specialist, as a textbook or handbook, rather than to toxicologists.

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. vii + 193. $16·80.

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. $16·80.

Residue Reviews: Residues of Pesticides and Other Contaminants in the Total Environment. Vol. 65. Edited by F. A. Gunther. Springer-Verlag, New York, 1976. pp. viii + 103. $16·00.

One of the major FAO/WHO projects in the field of data assimilation and expert assessment began in 1963 with the first Joint Meeting of the FAO Working Party of Experts on Pesticide Residues and the WHO Expert Committee on Pesticide Residues. Since then, the group has issued after each annual meeting a report of its conclusions and recommendations, followed by a separately published collection of monographs presenting a comprehensive survey of each of the pesticides discussed.

A summary of the principal data and conclusions on the carbamate and organophosphorus pesticides considered at the meetings held between 1963 and 1974 is presented in the first chapter of Volume 63 of 'Residue Reviews'. This is the second such review, the first having covered the organochlorine pesticides dealt with during the same meetings. The author is well qualified to present this material, having been a member of the Joint Meeting Secretariat for a number of years. He makes clear the criteria on which the toxicological evaluations of these carbamate and organophosphorus pesticides were based, before providing a list of the specific materials for which an ADI had been established by 1974. Short toxicological summaries then outline, for each pesticide, the main conclusions and comments and the available toxicity data and provide brief notes on uses, formulations and residues in foods. Altogether this constitutes a useful guide, permitting a quicker assessment of the position than can be derived from the detailed reports issued annually.

The second contribution to this volume is a much more restricted and detailed description of a single compound—the insecticide, Kelevan. The ethyl ester of a polychlorinated alicyclic carboxylic acid, Kelevan is in fact a reaction product of the perhaps more widely known Kepone with ethyl laevulinate. This survey of its uses, formulations, insecticidal potential, mammalian toxicity, environmental behaviour, residues on agricultural products and current tolerances and waiting times (in Germany) concludes that Kelevan is not comparable with the persistent chlorinated hydrocarbon insecticides in respect either of its toxicity or of its persistence in soils, plants and animals.

Finally, a lengthy paper, occupying over half of the book discusses the mass spectra of organophosphorus esters and their derivatives. Liberally provided with formulae and reproductions of the mass spectra of over 50 organophosphorus compounds, this review presents a wealth of data, backed by a list of more than 170 references.

Pesticide analysis also featured in Volume 64, in this case in relation to the general problem of residues. In particular. the reasons for organizing

collaborative studies and the ways in which this may be effected are considered. Some of the more recent results of collaboration are described in a fair amount of detail and the importance of sound analytical backing for the international tolerances now being established is stressed.

Another contribution in Volume 64 presents a topic of major interest to many toxicologists. It deals not only with nitrosamines in foodstuffs, as the title indicates, but also with the nitrosamine precursors present in the environment and the possible formation of nitrosamines *in vivo*. While written basically from the chemical standpoint, the review is not weighted unduly in favour of analytical aspects. It examines also the role of nitrate and nitrite in meat curing, identifies the problem of botulism and summarizes the chemical and biological properties of *N*-nitrosamines, before considering the occurrence and interaction of precursors in the environment and the actual identification of nitrosamines in specific foods. The author does stress the importance of further improvements in analytical techniques and more comparative testing of different methods, and he points out the necessity for further studies on the substances that catalyse or inhibit the nitrosation reaction. He also exhorts food technologists to continue the search for alternative ways of preserving food.

As has been the case with a few earlier volumes in this series, number 65 is in the form of a monograph. The subject in this instance is the action of triazine herbicides on the physiology of plants. This is a wide-ranging and detailed literature survey, backed by over 700 references and demonstrating the effects of the triazines on processes connected directly or indirectly with photosynthesis and with other energy-dependent reactions. Attention is also drawn to some of the questions that remain unanswered in this field.

Residue Reviews: Residues of Pesticides and Other Contaminants in the Total Environment. Vol. 66. Edited by F. A. Gunther. Springer-Verlag, New York, 1977. pp. viii + 212. $22·80.

The third, and presumably final, review in the useful series summarizing the conclusions and recommendations arising from the joint deliberations of the FAO Working Party of Experts on Pesticide Residues and the WHO Expert Committee on Pesticide Residue is featured in Volume 66 of *Residue Reviews*. Presented are toxicological data and evaluations from the 'Joint Meetings' of 1963–75 for eleven relatively small groups of pesticides, the organochlorine, carbamate and organophosphorus groups having been dealt with earlier. Relevant views of other international expert groups formed under the auspices of IARC and WHO are also considered. Readers are referred to the appropriate FAO, WHO and IARC reports for the original sources of the information cited, a procedure that restricts the list of references to workable proportions. Among the materials covered are some bipyridilium compounds, dithiocarbamates, chloronitrobenzene derivatives, dinitrophenols, diphenyls, isoindole derivatives, pyrethroids and organic compounds of mercury and tin. Many who are not strictly workers in pesticide toxicology, the group to which these reviews were directed, will be grateful for this guide to a lot of useful information.

The importance of polychlorinated biphenyls (PCBs) as persistent and widespread environmental contaminants has been clearly recognized in recent years, but work has been hampered by analytical problems, particularly in the separation of PCBs from other chlorinated hydrocarbon contaminants. Another contribution in this volume presents the case for using high-pressure liquid chromatography and flame-ionization or electron-capture detection, together with recently developed minicomputer facilities, for the qualitative and quantitative analysis of PCBs. In spite of a multiplicity of perhaps unavoidable abbreviations, this remains a very readable account of a problem and the prospects for overcoming it.

Another analytical contribution considers the methods available for the analysis of organotin compounds, particularly the residues of organotin pesticides. Finally two lengthy literature reviews deal with questions of major relevance to the ecological impact of pesticides in general. The first summarizes studies carried out over the last 30 years or so on the effects of pesticides on algal colonies, which are apparently not only highly susceptible to herbicides but are also affected by insecticides and fungicides. The second presents a 20-year survey of work on the capacity of anaerobic micro-organisms in a variety of ecosystems to metabolize different types of organic pesticides, on the identification of the metabolites and on the resistance of many compounds to such degradation. Both reviews suggest lines along which much further work needs to be directed.

Protein Crosslinking: Biochemical and Molecular Aspects/Protein Crosslinking: Nutritional and Medical Consequences. Edited by M. Friedman. Advances in Experimental Medicine and Biology, Volumes 86A and 86B. Plenum Press, New York, 1977. pp. xx + 760 & xx + 740. $59·50 per volume.

This monumental work records the proceedings of a Symposium on Protein Crosslinking held in San Francisco, California, from 30 August to 3 September 1976. To the material thus derived have been added various invited contributions. As a result, the two volumes constitute a detailed account of a subject that has never before been covered specifically, but that has emerged in recent years as one of the more critical areas in the fields of nutrition and biochemistry. The division of the two volumes under review into biochemical and molecular aspects, and nutritional and medical consequences, is a happy thought because it separates very clearly the basic research considerations from the practical aspects of great immediate importance and significance.

Throughout both volumes, one is struck by the very broad range of coverage, which includes both plant and animal systems. If any example were needed of the power, force and achievements of interdisciplinary research, these two volumes provide the evidence in ample measure. They are well-edited and well-

indexed works and are a striking example of a felicitous publication policy which has contributed many useful volumes in specialized subjects of wide general interest.

Membrane Toxicity. Advances in Experimental Medicine and Biology. Vol. 84. Edited by M. W. Miller and A. E. Shamoo. Plenum Publishing Corp., New York, 1977. pp. xiv + 553. $54·00.

This book contains the proceedings of the Ninth Annual Rochester International Conference on Environmental Toxicity held during May 1976 in Rochester, NY. The volume consists of 23 papers on five topics, together with the discussions and comments of the participants.

The first section, on xenobiotics and membrane transport, deals with various aspects of this subject, including the effects of ouabain, *p*-chloromercuribenzoate and a stilbene derivative on the intestinal transport of glucose, amino acids and sodium, the influence of heavy metals on ion permeability in the lung, and the membrane transport of antifolate drugs in normal and malignant cells. The second section consists of five papers concerned with the modification of membrane function by toxic compounds. This includes an excellent paper on the morphological and biochemical changes produced in the liver by carbon tetrachloride, and also one on membrane damage in radiation-induced cell necrosis. The third section, dealing with cellular responses to toxins, contains a paper on the toxic effects of silica on macrophages and T-lymphocytes and others on the changes in membrane permeability produced by lectins, ionophores and penicillin. The papers in the fourth and fifth sections are concerned with the use of molecular probes for investigating membrane conformation and function, and the interaction of compounds with receptor sites on membranes.

This collection of papers will doubtless be useful to workers thoroughly familiar with the subject but it could be of only very limited value to the general reader new to this specialized field.

Directory of On-going Research in Cancer Epidemiology 1977. Edited by C. S. Muir and G. Wagner. IARC Scientific Publications no. 17. International Agency for Research on Cancer, Lyon, 1977. pp. xi + 599. Sw.fr. 25·00 (available in the UK through HMSO).

The latest directory of cancer epidemiology studies reports on 908 as yet unpublished projects, a considerable escalation on the contents of the 1976 edition. Projects are listed according to their country of origin and encompass a total of 70 countries. The proportion of studies coming from developing countries has increased, although Europe, North America and Australia still play the major role.

The content of the directory reflects an increased interest in specific industrial hazards (e.g. vinyl chloride). Efforts are also focused chiefly on cancers of organs such as the lung, breast and uterus, while relatively little attention is given to other common cancers, such as those of the pancreas and prostate gland.

An introduction explains the workings of the clearing house for "ongoing research in cancer epidemiology" and the use of the directory. In addition to the main list giving details of each project, individual studies can be traced by means of indexes based on principal investigators and collaborators, cancer sites, aetiological factors and other relevant terms or key words, types of study (e.g. immunology, retrospective) and the country in which the study is being conducted. Population-based cancer registries are also listed.

Undoubtedly the continuation of this comprehensive directory is invaluable to those involved in the field of cancer epidemiology.

IARC Monographs on the Evaluation of the Carcinogenic Risk of Chemicals to Man. Some Miscellaneous Pharmaceutical Substances. Vol. 13. IARC Working Group. International Agency for Research on Cancer, Lyon, 1977. pp. 255 Sw.fr. 30·00.

The thirteenth set of monographs in this series is devoted to the evaluation, for the first time, of a group of established drugs (22 in all). It is worth repeating that these monographs summarize the evidence for the carcinogenicity of individual chemicals, but make no recommendations concerning preventive measures or legislation, since these matters depend on risk–benefit evaluation.

One of the strengths of these IARC monographs has been the fully comprehensive cumulative index presented in each volume and listing alphabetically all compounds considered to date, together with appropriate synonyms. It is therefore a little disappointing to note that eleven of the drugs considered by the Working Group at their thirteenth session were disregarded, on the grounds that "available data were inadequate". These drugs were acetylsalicyclic acid, amobarbital, chlorpromazine, diphenylthiohydantoin, LSD, nitroxoline, pentobarbital sodium, prednisone, pyrazinamide, rifampicin and spironolactone. It is to be hoped that some mention will be made of such compounds in the cumulative index of future volumes.

As in previous monographs in the series, very little guidance is given on the significance of the animal data presented. Either the limitations of existing information precluded any evaluation of carcinogenic risk, as was the case with acriflavinium chloride, chloroquine, 8-hydroxyquinoline and phenylbutazone, or evidence suggestive of carcinogenicity has been cited. For three of the compounds evaluated, namely oxymetholone, phenacetin and phenytoin, human data suggested that an increased risk of cancer might be associated with administration of the drug. Only in the case of phenytoin, however, was it possible to confirm some of these effects by recourse to data generated in animal experiments. The following compounds were carcinogenic in at least one animal species: aurothioglucose, oxazepam, dithranol (a tumour promoter), ethionamide, hycanthone mesylate, metronidazole, niridazole, phenobarbital sodium, phenytoin, pronetalol hydrochloride and pyrimetha-

mine. In all these cases, except metronidazole, niridazole and phenobarbital sodium, for which collateral data were obtained in hamsters or rats, the only evidence of carcinogenicity was generated in mouse studies. Even so, no mention is made by the Working Group of reservations that have been expressed in some quarters about the value of the mouse as a species for carcinogenicity testing.

Side Effects of Drugs Annual 2—1978. A Worldwide Yearly Survey of New Data and Trends. Edited by M. N. G. Dukes. Excerpta Medica, Amsterdam, 1978. pp. xix + 450. Dfl. 110·00.

The appearance of the second annual survey of the adverse effects of drugs presents the opportunity for a few additional comments on this ambitious and far-reaching project. The volume named above, like its predecessor published in 1977*, complements Volume VIII of the well-known *Meyler's Side Effects of Drugs* published in 1975 (*Cited in F.C.T.* 1977, **15**, 241). While revised editions of the latter, providing an overall review of the general situation relating to the adverse effects and interactions of drugs, are expected to appear about every 3–4 years, the annual volumes are intended to give a detailed and critical account of relevant new information that has appeared within a specified time. The first 'Annual' covered the field from January 1975, when the coverage of 'Meyler VIII' terminated, to 31 July 1976. The second covered the period from 1 August 1976 to 31 July 1977, and it is intended that volumes for succeeding years will present information appearing over this range of months. The deadline is not rigid, however, and later papers will be included where possible.

The three indexes follow the same useful pattern in all three books, guiding readers to the contents *via* the names of drugs or groups of compounds, their synonyms or the types of side effect produced. The chapters into which the text is divided are largely the same in both Annuals, and readers familiar with the 1977 edition will have no difficulty in finding their way around its successor, since the chapters are arranged and numbered in the same way in both volumes. Two new contributions—on lithium compounds and on drugs used in the management of gallstones—have been added in 1978, however. The helpful system of coding references to indicate the type or extent of the information provided (detailed review, brief or detailed original clinical evidence and so on) has been continued in the latest volume. Moreover, the cumulative indexing of the Annuals over each 4-year period, at the end of which a revised edition of 'Meyler' is scheduled to appear, means that all the information included on a given topic can be traced from only two indexes—those in the most recent edition of 'Meyler' and in the latest Annual.

Altogether much thought about the practicalities of their use seems to have gone into these compilations, as well as a great deal of information.

* *Side Effects of Drugs Annual 1—1977. A Worldwide Yearly Survey of New Data and Trends.* Edited by M. N. G. Dukes. Excerpta Medica, Amsterdam, 1977. pp. xvii + 420. Dfl. 100·00.

A Dictionary of Life Sciences. Edited by E. A. Martin. Macmillan Press Ltd., London, 1976. pp. 374. £5·95.

Elsevier's Dictionary of Food Science and Technology. Compiled by I. D. Morton and C. Morton. Elsevier Scientific Publishing Company, Amsterdam, 1977. pp. 207. D.fl 73·00.

Toxicology is no respecter of disciplines. Not only are those involved directly in toxicological research or consultancy obliged to view their problems from several different scientific angles, but they must be prepared to understand and take into account the technological background to many of their investigations. On the other side of the coin, the industrial chemist or technologist concerned with the safety of products or workers will inevitably become involved with biological studies and their terminology. Add to this the international connexions common to any science and dictionaries of one sort and another are likely to come into their own.

Two of the more recent of a long line of such publications are named above. The first encompasses the major aspects of botany and zoology, from anatomy and physiology to genetics and ecology, together with some biochemistry, endocrinology, animal behaviour and other more specialized branches of animal and plant study. The terminology of the applied biological sciences, such as medicine and toxicology, is largely excluded; the seeker of granuloma, sarcoma, LD_{50} and Heinz bodies, must, quite rightly, look elsewhere, but endoplasmic reticulum, oxidative phosphorylation, hyperplasia and autophagy are there, and within its chosen field the book presents a set of generally clear definitions. These are backed in many cases by explanatory diagrams and formulae, and in each explanation the existence of a separate entry for any of the terms used is clearly indicated.

Obviously the specialist may disagree with some aspects of the selection or with the extent or content of individual items—but the book is not intended for him. Designed for students, teachers and science journalists, among others, it contains much that could be useful to the 'non-biological technologist' who needs to follow the terminology of the many branches of biology on which toxicology impinges. To those obliged to embark on such unfamiliar seas (perhaps the "variegated scientists" named in a recent advertisement) this dictionary can hardly fail to be helpful.

The compilers of the second book named have provided the French, Spanish and German equivalents for a wide variety of nouns and some verbs used in the food and food-processing industries. The arrangement is simple. The first section consists of an alphabetical list of 2087 English words, each numbered and accompanied by the equivalent terms in the other three languages, while in the following French, Spanish and German sections each alphabetically listed word carries a number relating it to the English and other equivalents to be found in the first section. Finally, for good measure, any Latin terms appearing in the first (English) section are listed, with the relevant numbers. While many of the terms included are no doubt to be found in good French, German or Spanish chemical or even general dictionaries, there

are numerous others that would be difficult to find, or might well be inadequately translated, elsewhere.

BOOKS RECEIVED FOR REVIEW

Criteria (Dose/Effect Relationships) for Cadmium. Report of a Working Group of Experts prepared for the Commission of the European Communities, Directorate-General for Social Affairs, Health and Safety Directorate. Rapporteur R. Lauwerys. Pergamon Press, Oxford, 1978. pp. vii + 202. £9·50.

Residue Reviews: Residue of Pesticides and Other Contaminants in the Total Environment. Vol. 68. Edited by F. A. Gunther. Springer-Verlag, New York, 1977. pp. viii + 154. $19·80.

A Colour Atlas of Histological Staining Techniques. By A. Smith and J. Bruton. Wolfe Medical Publications Ltd., London, 1977. pp. 192. £5·00.

A Colour Atlas of Neuropathology. By C. S. Treip. Wolfe Medical Publications Ltd., London, 1978. pp. 208. £15·00.

Environmental Health Criteria 4. Oxides of Nitrogen. Published under the joint sponsorship of the United Nations Environment Programme and the World Health Organization. WHO, Geneva, 1977. pp. 79. Sw.fr. 9·00 (available in the UK through HMSO).

Drug Metabolism—From Microbe to Man. A Symposium in Honour of Richard Tecwyn Williams. Edited by D. V. Parke and R. L. Smith. Taylor & Francis Ltd., London, 1977. pp. xii + 460. £25·00.

Microbial Ecology of the Gut. Edited by R. T. J. Clarke and T. Bauchop. Academic Press Inc. (London) Ltd., 1977. pp. xvii + 410. £13·50.

Fd Cosmet. Toxicol. Vol. 16. pp. 491–496. Pergamon Press Ltd 1978. Printed in Great Britain

ARTICLES OF GENERAL INTEREST

TRICHLOROETHYLENE: HEPATIC EFFECTS, METABOLISM AND ELIMINATION

The potential toxicity of trichloroethylene (TRI) in industrial situations is well known, as is the importance of inhalation as a route of entry into the body. Several studies in man have identified trichloroethanol (TCE) and trichloroacetic acid (TCA) as major metabolites (*Cited in F.C.T.* 1973, **11**, 1154) and chloral hydrate as a transient intermediate in the metabolic process (*ibid* 1976, **14**, 158). TCE has been widely regarded as the metabolite responsible for the toxic effects of TRI (*ibid* 1974, **12**, 163). Various attempts have been made to relate levels of TRI exposure to elimination rates to provide a basis for the monitoring and control of occupational exposure, and methods suggested for estimating levels of exposure have included the simultaneous determination of the individual metabolites of TRI in the blood, using a gas-chromatographic microtechnique (*ibid* 1973, **11**, 1154), and the determination of respiratory elimination rates (*ibid* 1975, **13**, 591).

Nevertheless, the relationship between the degree of exposure and the excretion of the solvent and its metabolites has remained difficult to define. In an attempt to clarify the situation, Fernandez *et al.* (*Br. J. ind. Med.* 1977, **34**, 43) have studied the absorption, distribution and elimination of TRI and the kinetics of the formation and excretion of its metabolites by means of a mathematical model. The results indicate that both post-exposure breath decay curves and urinary elimination curves of TCE and TCA serve to monitor time-weighted average exposures, but while urinary excretion of TCE reflected the most recent exposure, that of TCA was related to the average exposure of preceding days. Pulmonary elimination studies during single or repeated exposures showed a linear relationship between the alveolar concentration of TRI about 15 hours after cessation of exposure and the amount of the solvent deposited in fatty tissues. Thus under industrial conditions, the choice of monitoring test must depend on the type of exposure and the information required, while the timing of sample collections must be determined by the kinetics of elimination of the selected indicator.

Sato *et al.* (*ibid* 1977, **34**, 56) also resorted to mathematical models, estimating the rate constants for TRI absorption, distribution, excretion and metabolism from the time-course data of the solvent concentration in blood or from the urinary excretion of its metabolites. Using data from an exposure test in which four men inhaled 100 ppm TRI for 4 hours, both the model for the blood-concentration data and that for urinary excretion were found to give good estimates of the actual rate constants for the transfer of TRI in the body. TCA accounted for about 20% of the total trichloro compounds excreted in the urine after this single exposure, and TCE for the rest, but as TCA was shown to have a longer biological half-life than TCE, it is likely to account for a greater proportion of the urinary metabolites after chronic exposure to TRI.

Relating the hepatotoxicity of TRI to its metabolism, Moslen *et al.* (*Biochem. Pharmac.* 1977, **26**, 369) found that rats anaesthetized for 2 hours with 1% TRI developed acute liver injury if they were pretreated with mixed-function oxidase inducers, including phenobarbitone, Aroclor 1254, hexachlorobenzene, 3-methylcholanthrene and pregnenolone-16-α-carbonitrile, but not if they received spironolactone or only the inducer vehicle. Phenobarbitone and Aroclor 1254 were the inducers associated with the most severe liver injury, the morphological changes being accompanied by a marked electrolyte disturbance and a more than 20-fold rise in serum-transaminase levels. The latter rise was directly proportional to the prolongation of recovery from the anaesthesia and the enhanced excretion of metabolites. Further studies confirmed a direct correlation between the mean content of cytochrome *P*-450 at the start of the TRI exposure and the extent of liver injury, as judged by the serum-transaminase level, prolongation of recovery time after anaesthesia and enhancement of urinary excretion of trichlorinated metabolites. In another study, Moslen *et al.* (*Res. Commun. chem. Path. Pharmac.* 1977, **16**, 109) showed that in fasted rats exposed to 1% TRI for 2 hours after premedication with phenobarbitone, cytochrome *P*-450 and cytochrome b_5 were decreased after the first hour of TRI exposure, and NADH-cytochrome *c* reductase was increased threefold after 8 hours. In contrast to control animals, in which hepatic glutathione (GSH) remained constant during exposure to TRI but rose later, liver GSH was reduced in phenobarbitone-treated animals during TRI exposure and rebounded later, the maximal decrease occurring in the microsomal fraction. Feeding rats increases hepatic GSH levels, compared with the fasting situation, without in itself affecting the capacity for mixed-function oxidase activity. In fed rats exposed to TRI, anaesthesia-recovery time was shorter and liver injury was less severe than in fasted animals similarly exposed to TRI, although urinary excretion of trichlorinated metabolites over the next 24 hours was similar to or slightly more than that in the fasted animals. This work thus provides some support for the view that the hepatotoxicity of TRI may derive from inadequate detoxification of its reactive intermediates.

It has been suggested that the hepatic tumours reported in mice given oral doses of TRI (*Food Chemical News* 1975, **17** (15), 36) may be attributable to an epoxide intermediate (*Cited in F.C.T.* 1977, **15**, 85). Incubation of TRI with strains of *Escherichia coli*

demonstrated no mutagenic activity, but the incorporation of a metabolizing microsomal-enzyme preparation into the incubation system resulted in some increase in the mutation rate (*ibid* 1977, **15**, 85). The National Cancer Institute studies mentioned above demonstrated a carcinogenic effect in mice but not in rats treated orally with TRI (US Department of Health, Education, and Welfare; National Cancer Institute; Carcinogenesis Bioassay of Trichloroethylene; Carcinogenesis Tech. Rep. Ser. no. 2, Washington, DC). Nevertheless, Van Duuren & Banerjee (*Cancer Res.* 1976, **36**, 2419) have shown that when microsomal preparations from rat liver were incubated with ^{14}C-labelled TRI, the label became covalently bound to microsomal protein. Binding was decreased by incubation with 7,8-benzoflavone and blocked completely by SKF-525A, two known inhibitors of mixed-function oxidases, and was enhanced by pretreatment of the rats with phenobarbitone. The addition of 3,3,3-trichloropropene oxide, a powerful epoxide-hydratase inhibitor, to the incubation system markedly enhanced the binding of TRI to microsomal protein, but protein binding was diminished by addition of urea, GSH, 1-methyl-2-mercaptoimidazole or mercaptoethanol to the system. These results indicate that an epoxide or related electrophilic intermediate is involved in the binding of TRI to microsomal proteins in the rat liver.

Uehleke *et al.* (*Arch. Tox.* 1977, **37**, 95) have provided spectral evidence for the formation of an epoxide, 2,2,3-trichloro-oxirane, during the microsomal metabolism of TRI. Aerobic incubation of TRI with rabbit-liver microsomes and NADPH produced a metabolite with a difference absorption peak at 451–452 nm. A similar peak was observed when 2,2,3-trichloro-oxirane was added to microsomal preparations, and was attributed to rearrangement of the epoxide to dichloroacetyl chloride, which produces an absorption change in the region of 452 nm. The difference absorption produced by the latter compound, however, was only some 20% of that produced by the epoxide in a comparable concentration. The TRI epoxide can be rearranged either to dichloroacetyl chloride or to chloral, the former conversion being favoured by nucleophilic agents, such as tertiary amines, and the latter by electrophilic compounds, including Lewis acids. Henschler *et al.* (*ibid* 1977, **37**, 233) suggest that while a Lewis acid-type catalytic rearrangement to the nonreactive chloral hydrate seems likely to occur in the mammalian liver cell, this reaction may not necessarily predominate in micro-organisms used as tester strains in *in vitro* mutagenicity assays.

These authors (*loc. cit.*) also point out that technical-grade TRI contains several impurities and additives, including antioxidants which are essential for the stabilization of TRI used as a degreasing agent. These contaminants may have significant biological activity. Gas chromatography and mass spectrometry of a sample of TRI undergoing carcinogenicity testing identified contaminants accounting for 0·65% of the sample. The suspected mutagens/carcinogens among these, epichlorohydrin, 1,2-epoxybutane and diisobutylene, were tested for mutagenicity in *Salmonella typhimurium* TA100. The first two compounds demonstrated high mutagenic activity, while that of the third was low. In the same test system, carbon tetrachloride, chloroform and 1,1,1-trichloroethane, which were also present in the TRI sample in concentrations of 0·01–0·05% (w/w), proved inactive, even in the presence of rat-liver microsomes. It seems possible, therefore, that the carcinogenic effect of some samples of technical TRI may be attributable, partly if not exclusively, to their content of added epoxides. Not all samples of even technical-grade TRI are treated with epoxides, but the possible importance of this factor, it is suggested, receives some support from the fact that the TRI analysis was carried out on the material that induced hepatic carcinomas not in rats but only in mice, a species in which the activity of epoxide hydratase is comparatively low.

[P. Cooper—BIBRA]

A FLAME RETARDANT DOUSED

The accidental contamination of fodder with a hexabrominated biphenyl (HBB) several years ago was followed by deaths, stillbirths and high abortion rates in the Michigan cattle given the feed (*Food Chemical News* 1974, **16** (10), 34). Polybrominated biphenyls (PBBs) have been shown to be excreted in cows' milk (*Cited in F.C.T.* 1977, **15**, 157) and to have marked effects on the mammalian liver (*ibid* 1976, **14**, 648; *ibid* 1978, **16**, 74 & 195). During the Michigan outbreak, neurological, muscular and gastro-intestinal disturbances among members of the local population were attributed to exposure to PBB, but the association was not proven (*Food Chemical News* 1974, **18** (43), 32).

Before this major disaster brought the toxicity of the PBBs into the public eye, the increasing demand for flame retardants for textiles and the potential value of several PBB compounds for flameproofing certain types of fibre had prompted a number of toxicological programmes designed to investigate the possible industrial, consumer and environmental hazards associated with specific compounds in this group. The data generated by one such programme before development work on the compounds was terminated have recently been published (Waritz *et al.* *Am. ind. Hyg. Ass. J.* 1977, **38**, 307). The studies reported were concerned with an octabrominated biphenyl (OBB) and to a limited extent with an HBB. The technical sample of OBB tested is stated to have contained 1% heptabromobiphenyl, 60% nonabromobiphenyl and 6% decabromobiphenyl and only 33% of the octabrominated compound.

In the acute experiments, the approximate lethal dose of this OBB in male rats exceeded 17 g/kg. Liver

enlargement was seen with single doses of 3·4 g/kg or more. In a 28-day feeding study, rats fed 1–1000 ppm OBB in the diet showed no changes in weight gain, food consumption or food efficiency, and there was no evidence of clinical toxicity. There was a slight increase in liver weights at feed levels of 100 and 1000 ppm. In these two groups the hepatocytes were markedly enlarged after the first 2 weeks of treatment, and the hepatocellular hypertrophy was accompanied by displacement of basophilic cytoplasmic granules towards the cell periphery, the appearance of laminated cytoplasmic inclusions, foamy or vesicular alteration of the perinuclear cytoplasm and depletion of hepatocellular glycogen, all these changes being apparent mainly in the centrilobular zone.

Rabbits given a 24-hour skin application of a 35% (w/v) OBB paste in oil showed no toxic signs, whereas application of the same concentration of HBB caused lethargy and loss of weight in the recovery period. OBB applications produced no gross pathological changes, but following HBB application in a dose of 5 or 10 g/kg, necrotic foci occurred in the liver, and fat stores were depleted. Intact rabbit skin to which 1 or 0·1 g OBB/kg was applied in corn oil under occlusion for 6 hours on 5 days in each of 2 weeks showed mild erythema and roughness. Applied as a 50% slurry in propylene glycol to intact guinea-pig skin three times weekly for 3 weeks, or by intradermal injection of a 1% (w/v) solution in dimethylsulphoxide on each of four successive weeks, OBB induced no sensitization. Polyester fabric containing 6% OBB applied to human skin was neither irritant nor sensitizing. No effect on the cornea or iris was evident after introduction of 100 mg OBB into the conjunctival sac of the rabbit, but unless the compound was washed out within 20 seconds, it caused mild conjunctival redness and swelling and a copious discharge, all of which disappeared within 4 hours.

Exposure of rats to 0·96 mg OBB dust/litre for 4 hours was not lethal, caused no significant increase in relative liver weight, and produced no microscopically detectable liver lesions within 7 days. Fumes from about 30 g OBB heated to 350°C killed one of six rats within 24 hours; the dense fumes produced a time-weighted atmospheric concentration of brominated material equivalent to 1400 μg Br/litre. Rats exposed to the fumes produced by heating OBB at 290°C for 4 hours daily for 10 consecutive days suffered no adverse reactions either during exposure or for 7 days thereafter. No histopathological effects were seen in the animals exposed to fumes at either temperature, but there was some increase in relative liver weight in both groups.

Rats fed 100, 1000 or 10,000 ppm OBB in the diet on days 6–15 of gestation showed no significant changes in the reproduction indices and no clear evidence of any teratogenic effect. Single instances of gastroschisis (fissure of the abdominal wall with protruding viscera) and of generalized oedema of the subcutaneous connective tissue among the foetuses exposed to the 10,000- and 1000-ppm levels of OBB may have been fortuitous. OBB was transferred to the foetus, but its presence and concentration in blood or organs was not investigated further.

Analysis of samples of liver, fat and muscle from rats killed at intervals during and after the treatment period in the 28-day feeding study revealed a significant increase in bromide content in all three tissues, and particularly in the fat and liver, even in the group fed the 10-ppm dietary level. As in the liver and fat of the dams in the teratogenicity study, the levels of bromide varied directly with the dietary level of OBB. Similarly, in an inhalation study included in the same programme, exposure of rats to approximately 3·5 pg OBB/litre air for 23 hours every day for 15 weeks had no detectable clinical or pathological effects but caused a significant rise in the levels of bromide in the fat and liver, although not, in this case, in the muscle tissue. These indications of the likely accumulation of OBB or its breakdown products in depot fat, combined with the difficulty of protecting workers adequately and the probable environmental effects of the material are reported by Waritz *et al.* (*loc. cit.*) to have been important factors in the assessment of OBB as unsuitable for large-scale use as a flame retardant for textiles.

In tests on the rabbit ear, a PBB containing predominantly HBB (Firemaster FF-1) produced chloracne (Kimbrough *et al. Lancet* 1977, **II**, 602). In rats given the same material in a single dose of 1 g/kg by gavage, females showed mean blood concentrations of 2·9 ppm HBB after 300 days and 2·92 ppm after 420 days; males showed means of 0·94 and 1·34 HBB, respectively, after the same periods. The mean toxic effect of HBB was on the liver, where lipid accumulation was pronounced in males 2, 6 and 10 months after dosing, and uroporphyrin accumulated in females. Under light microscopy, the livers of the dosed rats showed enlarged hepatocytes with foamy or vacuolated cytoplasm, general pleomorphism and brown-pigment accumulations in the macrophages and Kupffer cells. Isolated liver cells showed interstitial fibrosis and necrosis, particularly in females. Small neoplastic nodules, as defined by Kimbrough *et al.* (*J. natn. Cancer Inst.* 1975, **55**, 1453), were seen in the livers from about half of the rats killed 10 or 14 months after HBB treatment, the females again being most commonly affected. Thus it appears that HBB is very slowly eliminated from the rat, and that the persistent liver lesions it produces may progress to neoplasia. Kimbrough *et al.* (1977, *loc. cit.*) point out, however, that this prolonged retention may be due either to enterohepatic circulation or to the rat's inability to excrete PBBs. Moreover, it has yet to be established whether enterohepatic recirculation of PBBs occurs in man, whether PBBs are toxic to the human liver at blood levels that are effective in the rat, and whether they are metabolized in a similar fashion in rats and man. On the basis of animal observations, high exposures of man to PBBs should prompt a search for hepatic porphyria, and more research is needed to determine whether a contaminant or any specific isomer present in a PBB mixture is primarily responsible for the toxic effects observed in animals (Kimbrough *et al.* 1977, *loc. cit.*).

In a study of the effect of a PBB mixture on hepatic and mammary enzyme activities in lactating rats, Dent *et al.* (*Life Sci.* 1977, **20**, 2075) fed a PBB containing 70% HBB (Firemaster BP-6) to female rats at a dietary level of 50 ppm from day 8 of gestation to day 14 after parturition. The growth rate of the dams was not affected by the treatment, but the liver-

to-body weight ratios increased significantly. In the liver, microsomal protein was increased and microsomal arylhydrocarbon hydroxylase (AHH) and epoxide hydratase (EH) activities were increased by 980 and 230%, respectively. Mammary gland AHH and EH activities also changed significantly, the former increasing by 200% and the latter falling by 50%. The ratio of mammary weight to body weight and the quantity of mammary protein did not alter significantly.

The full implications of these enzyme changes cannot be clearly defined at present. However, it is likely that an alteration in the relative activities of AHH and EH could affect the amount of a reactive intermediate available to initiate a toxic response, since AHH activity is credited with the formation of reactive electrophilic intermediates from certain relatively inert precursors, while EH converts these arene oxides to less reactive compounds. The changes demonstrated in the mammary-gland enzymes could, therefore, have important repercussions with regard to foreign-compound activation and deactivation, not only in the treated dams but also in their suckling pups.

There is apparently some tendency for any current problems on Michigan dairy farms to be attributed to the aftermath of the 1973 contamination of feed with PBB. In an attempt to contribute some facts to the inevitable debates on this question, Moorhead *et al.* (*J. Am. vet. Med. Ass.* 1977, **170**, 307) administered a commercial PBB mixture (Firemaster BP-6) to three groups of six pregnant heifers in daily doses of 0·25, 250 or 25,000 mg (equivalent initially to about 0·006, 0·65 and 67 mg/kg body weight day) for 60 days or until the animals became moribund. In the group receiving the highest dose, anorexia, excessive lachrymation and salivation, diarrhoea, emaciation, dehydration, depression and abortion occurred, and the animals had to be killed for autopsy after 33–66 days on the test diet. In the groups receiving 0·25 mg or 250 mg PBB daily, no toxic signs developed and no adverse effects were detected at autopsy by gross or histopathological examination.

In the maximally exposed heifers, serum glutamic–oxalacetic transaminase and lactate dehydrogenase rose to a peak at about the time of foetal death or abortion, and were thus of little diagnostic value. Similarly, blood-urea nitrogen values began to rise only after about day 15 and were of little help in the early diagnosis of kidney damage, although Moorhead *et al.* (*loc. cit.*) suggest that these values may be useful as a screening procedure to determine whether cattle have been exposed to toxic quantities of this PBB mixture. Terminal urine analyses revealed a slight drop in specific gravity and pH, and some proteinuria. Slight increases in serum-bilirubin values reflected gall-bladder changes and minimal hepatocellular damage detected at autopsy. The main changes demonstrated at that stage, in addition to gall-bladder hyperplasia and cystic dilatation of the mucous glands in the lamina propria, were extreme dilatation of the collecting ducts and tubules of the kidney, with epithelial degenerative changes involving cloudy swelling, hydropic degeneration and separation from the basement membrane. In addition, hyperkeratosis was apparent in the eyelids, with accumulations of keratin in hair follicles of the epidermis and squamous metaplasia with keratin cysts in the tarsal glands.

The doses of PBB given to the three groups in this study were those calculated to achieve body-fat concentrations of PBB ranging approximately from the current FDA tolerance of 0·3 ppm (calculated on fat) to the high levels that resulted initially from the contamination incident. While the non-specific clinical signs (anorexia, dehydration, excessive lachrymation, salivation and diarrhoea) observed in the cattle given the highest level of treatment were reported also among the animals accidentally contaminated in Michigan, eyelid hyperkeratosis and histopathological changes in the renal tubules and gall bladder have not been consistently reported, although they may have been present in the animals initially contaminated. In view of the findings of this study, the authors of the report suggest that the adverse effects currently being reported among Michigan cattle, in some cases in those with tissue residues of PBB well below the FDA guideline, may in fact be a reflection of very different factors, such as nutritional deficiencies or indigenous microbial or parasitic infections.

[P. Cooper—BIBRA]

CARBON DISULPHIDE: STILL SOME WAY TO GO

The mechanism or mechanisms responsible for the wide-ranging toxic action of carbon disulphide (CS_2) are still in doubt, in spite of an impressive volume of research. What is not in doubt, unfortunately, is the significant contribution the material has made over the years to the ill-health and mortality of workers involved in the production of viscose rayon. Whilst conditions in this industry have been markedly improved since the war, it is still uncertain whether 'safe' industrial limits have yet evolved. Data from the more recent epidemiological studies on CS_2, summarized in this review, suggest that further improvements may be necessary.

The results of a prospective survey at the Finnish Institute of Occupational Health of 343 workers in a viscose-rayon plant over the period 1967–72 were briefly summarized in our last review on CS_2 (Potter, *Fd Cosmet. Toxicol.* 1977, **15**, 243). In an extension of this study, the time of observation has been increased to 8 years, and now covers the period up to 1975 (Hernberg *et al. Scand. J. Work. envir. Hlth* 1976, **2**, 27; *Nurminen, Int. J. Epidemiol.* 1976, **5**, 179). Those workers with a history of exposure to CS_2 were still twice as likely to die from coronary heart disease than were a matched control group. Even excluding heart disease as a cause of death, there was a 2-year

difference in the life expectancy of the two groups for the age interval 25–54 years. Since 1972, conditions within the plant have been improved to give atmospheric concentrations of CS_2 below 10 ppm. During the period 1972–75, no excess mortality occurred in the group with a history of industrial exposure to CS_2, but only 19% of the original sample of 343 workers were still in daily occupational contact with the solvent. In 1972, 53% of the original sample were still working with CS_2.

The same group of Finnish epidemiologists has collaborated with Japanese counterparts to study further the effect of CS_2 on the heart (Tolonen *et al. Int. Archs occup. envir. Hlth* 1976, **37**, 249). In this combined investigation, two groups of exposed workers, one Finnish (237 men) and the other Japanese (417 men) were compared with a roughly equal number of controls of the same nationality. Factory conditions were comparable in the Finnish and Japanese rayon plants, atmospheric concentrations of CS_2 decreasing from the 20–60 ppm of the 1950s down to 5–10 ppm in the 1970s. There were no significant differences in the incidence of electrocardiogram abnormalities within the exposed as compared to the unexposed groups. Whilst the prevalence of angina pectoris and the blood pressure of the Japanese men exposed to CS_2 did not differ markedly from those of unexposed controls, the Finnish exposed workers did experience anginal pains more frequently and have significantly higher diastolic and systolic blood pressures than their controls.

As regards the toxic effect of CS_2 on the heart, the results obtained at the Finnish Institute are reassuring. Conditions currently found in the Finnish viscose-rayon industry seem to have resulted in a significant reduction in the hazards of the workplace. The small number of individuals still subject to the toxin, together with the lack of sensitivity of mortality rates as an indicator of health mean that the encouraging results require confirmation. Signs of cardiac toxicity were still observed in the Finnish workers although admittedly not in the Japanese: neither group had been exposed regularly to CS_2 levels in excess of 30 ppm since 1960. Therefore, it is certainly not possible at present to state that regular exposures to low levels of CS_2 are without hazard.

Previously epidemiological studies of workers in the Japanese viscose-rayon industry have indicated that chronic exposure to CS_2 may produce microscopic aneurysms in the retina. As many as 31% of a workforce subjected to poor working conditions were shown by Sugimoto *et al.* (*ibid* 1976, **37**, 1) to be affected. The same investigators have also studied the condition of the retina in 214 workers (drawn from 11 plants with widely differing conditions) over a 5-year period (*idem. ibid* 1976, **37**, 233). Their original sample included 58 cases of retinopathy. The area affected increased in 23% of the group exposed to CS_2 throughout the period of the experiment, whereas only 13% of the group removed from the working environment sometime during the 5 years suffered a similar change. No deterioration was observed in any worker with a total history of exposure to CS_2 of less than 10 years. Complete or partial improvement in the condition of the retina occurred in only 1·5% of the exposed group but in 11·3% of the 'removed group', and improvements were noted even in individuals who had left the workplace after a long history of CS_2 exposure (exceeding 10 years). Previously epidemiology conducted in both Japan (*Cited in F.C.T.* 1973 **11**, 151) and Italy (*Ferrero, Medna Lav.* 1969, **60**, 38) has incriminated CS_2 as a possible cause of diabetes: abnormalities of the retina are sometimes associated with a disturbed glucose metabolism. Although in the study described above higher blood-glucose levels were noted in workers with retinopathy, no correlation between retinopathy and diabetes was found.

The earliest reports of the industrial hazards associated with CS_2 appeared before the last war and featured almost exclusively neurotoxic effects of varying severity. Peripheral neuropathy has continued to be observed in workers involved in the production of viscose rayon and thus exposed to average CS_2 levels below 30 ppm (Seppäläinen *et al. Work Envir. Hlth* 1972, **9**, 71). Electrophysiological examination of the peripheral nerves of a group of 118 male workers who had a history of 1–27 years (median 15 years) exposure to CS_2 was undertaken by Seppäläinen & Tolonen (*ibid* 1974, **11**, 145). The members of this group, drawn from the workers being monitored by Hernberg *et al.* (*loc. cit.*) for mortality from heart attacks, were involved in both viscose-film and rayon-filament manufacture. In both cases, the plant-room concentrations of CS_2 had seldom exceeded 30 ppm since the 1960s. It was found that 48% of the exposed group, compared with 24% of a control group of 100 men, had polyneuropathy ($P < 0{\cdot}001$), defined by Seppäläinen & Tolonen (*loc. cit.*) as a pathologically reduced conduction velocity in two or more nerves (eight nerves being measured for each subject). The motor nerves in the arms and legs were affected by CS_2 exposure, although the neuropathy was more pronounced in the legs. As cessation of exposure did not lead to any noticeable increase in the velocity of nerve conduction, even in individuals who had not worked with CS_2 for 10–15 years, the observed subclinical polyneuropathy was probably irreversible. In a further electroencephalographic examination, 21 of the group of 54 exposed workers compared with six of the 50 controls had abnormalities in their electroencephalograms.

Vasilescu (*Revue Roum. Neurol.* 1972, **9**, 63) also demonstrated that the conduction velocity of the peripheral motor nerves was significantly reduced in subjects with chronic CS_2 poisoning, even at an early stage of neuropathy when only subjective symptoms were present. Whilst Seppäläinen & Tolonen (*loc. cit.*) only reported effects on the motor nerves, Vasilescu (*Eur. Neurol.* 1976, **14**, 447) noted that the conduction velocity of the sensory fibres of the arms and legs was also affected by CS_2. Indeed he considered that the electrophysiology of the sensory nerves provided the more sensitive measure of the health of the peripheral nervous system. In a group of 81 individuals suffering from CS_2 poisoning, he found the most significant reduction in conduction velocity in the 30 patients with objective signs of poisoning, such as glove hypoaesthesia. However, even in 21 patients with only subjective symptoms of poisoning and motor-nerve conduction velocity within normal limits, the conductivity of the sensory nerve fibres in

both the arms and legs was significantly reduced, especially in the distal segments. In the study of Seppäläinen & Tolonen (*loc. cit.*), the sensory nerves of the arm were unaffected by CS_2 exposure; those of the leg were not examined. The data of Vasilescu (1976, *loc. cit.*) suggested, therefore, that a diagnosis of polyneuropathy could always be made in the subclinical stage. His results, while correlating the severity of the clinical picture with the electrophysiology of the nerves, also indicated that the polyneuropathy elicited by CS_2 was due to a dying-back lesion, changes in conductivity occurring first in the terminal segment of the long nerves possibly as a result of axonal degeneration.

The TLV for CS_2 in workroom atmospheres is now set at 20 ppm in the UK and USA. On the basis of the results outlined above, it seems that regular occupational exposure to this level of CS_2 may still pose a threat to the health of a workforce. Certainly the present standards of industrial hygiene in Finnish, Hungarian and Japanese viscose-rayon plants have not prevented the development of either neurotoxicity, possibly irreversible in nature, or eye lesions in a number of exposed workers.

[J. Hopkins—BIBRA]

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TOXICOLOGY: BIBRA ABSTRACTS AND COMMENTS

COLOURING MATTERS

3321. Tartrazine and the nervous system

Sobotka, T. J., Brodie, R. E. & Spaid, S. L. (1977). Tartrazine and the developing nervous system of rats. *J. Toxicol. envir. Hlth* **2**, 1211.

Organic colouring agents are amongst the additives accused, rather equivocally, of being responsible for the hyperkinetic behavioural syndrome in children (*Cited in F.C.T.* 1977, **15**, 357). Tartrazine has also been implicated in cases of food allergy (*ibid* 1973, **11**, 685).

Pregnant rats were started on a diet containing 1 or 2% tartrazine from day 7 of gestation. After weaning, the offspring were maintained for the remainder of 3 months on the diet given to their dams, and were then tested for righting reflex, neuromotor clinging ability, auditory startle response, placing response and general motor activity. The dams were tested for changes in motor activity immediately after the pups were weaned.

Tartrazine did not affect maternal body weight, although there was a transiently increased consumption of tartrazine-containing diets over control diet during wk 1, and no changes in maternal motor activity were apparent after the pups were weaned (21 days after birth). Average litter size, birth weights and survival rate at wk 3 did not differ between test and control groups, but both male and female pups had significantly reduced body weights after taking the tartrazine diets. This effect reached a nadir of 13–14% reduction 21 days after birth in males, but in females was only significant at 36 and 93 days of age. Absolute weights of brain and adrenals of the pups at weaning were unchanged by the treatment, but thymus weight was significantly depressed. Increased relative brain and adrenal weights reflected the reduced body weights in the males. The red blood cell counts and haemoglobin concentrations were slightly elevated in the group on 2% tartrazine. No effects of tartrazine on neuromotor function were observed in the postnatal period, except that the clinging ability of female pups was slightly increased on days 4–8. The age at which the eyes opened was unaltered.

Brain chemistry, as depicted by the regional distribution of protein, cholesterol and DNA, and the motor activity of weanlings were not affected by tartrazine exposure.

PRESERVATIVES

3322. Bacterial mutagens from smoked foods

Nagao, M., Honda, M., Seino, Y., Yahagi, T. & Sugimura, T. (1977). Mutagenicities of smoke condensates and the charred surface of fish and meat. *Cancer Lett.* **2**, 221.

For many years an association has been alleged between a high consumption of smoked foods and stomach cancer (*Cited in F.C.T.* 1970, **8**, 244). One fact frequently mentioned in support of this contention is that a major dietary source of the potent carcinogen benzo[*a*]pyrene is smoked fish and meat (*ibid* 1972, **10**, 571).

Condensates of smoke collected during the broiling of fish and extracts from charred fish and meat have been examined for mutagenic activity in *Salmonella typhimurium* strains TA100 and TA98, both in simple culture and in the presence of the metabolizing S-9 fraction of rat-liver homogenate. The dimethylsulphoxide (DMSO) extracts of smoke condensates from broiled herring (*Clupea pallasi*) and mackerel pike (*Cololabis saira*) were mutagenic in both strains in the presence of S-9 fraction, and that from mackerel pike showed fairly strong mutagenic activity without S-9 activation. The smoke condensate of sardine was mutagenic in TA98 after activation but its mutagenicity in TA100 was very weak. DMSO extracts of the charred skin of sardine and horse mackerel (*Trachurus japonicus*) were strongly mutagenic for TA98 in the presence of S-9 fraction. DMSO extracts of smoke condensate from charcoal-broiled beefsteak were weakly mutagenic towards TA98 but not TA100 both with and without activation, and an extract of charred meat was also mutagenic towards TA98, this mutagenicity being enhanced by metabolic activation. The benzo[*a*]pyrene equivalents calculated from the mutagenicities of both smoke condensates and charred fish and meat extracts were much higher than might have been expected from estimates of their actual benzo[*a*]pyrene contents, being 132 μg for smoke from 100 g of sardine, 358 μg for charred sardine surface and 855 μg for 190 g of beefsteak surface. The active mutagenic agent in these tests thus requires further investigation, as does the question of whether this activity is paralleled by carcinogenic activity in more sophisticated studies.

MISCELLANEOUS DIRECT ADDITIVES

3323. Fate of ingested tartrate isomers

Lewis, J. D. (1977). Comparison of the distribution of L(+) and DL-forms of tartaric acid in the rat. *Acta pharmac. tox.* **41**, Suppl. I, 144.

Tartaric acid, often in the form of its sodium salt, is added to a variety of foods, including soft drinks, confectionery and bakery products. While L-tartaric acid occurs widely in nature, the usual commercial processes of synthesis yield the racemic (DL-) form.

In rats treated on seven successive days with ^{14}C-labelled L(+)- or DL-tartrate in an oral dose of 2·73 mg/kg, radioactivity was present mainly in the gastro-intestinal tract, kidneys, liver and the inorganic bone matrix 3 hr after the last dose. In the kidney, activity derived from DL-tartrate was associated with 'granular' material in the cortex and medulla, whereas that derived from L(+)-tartrate appeared to be evenly distributed throughout the cortex. The radioactive granular material remained in the kidneys 24, 48 and 192 hr after the final administration of DL-tartrate, but no renal radioactivity remained at these times after the last dose of L(+)-tartrate. In other body tissues, radioactivity from both isomers showed a parallel pattern of decline, and in inorganic bone, activity remained 192 hr after the last doses.

Renal retention of radioactivity was probably attributable to the precipitation of poorly soluble calcium DL-tartrate in the tubules, the more soluble calcium L(+)-tartrate being removed rapidly. Similarly, plasma radioactivity diminished more rapidly after the dose of L(+)-tartrate than after that of DL-isomer, and 6–24 hr after the final dose the radioactivity in bone was about twice as great for DL- as for L(+)-tartrate.

It follows that in feeding tests with tartrates, results obtained with the naturally occurring laevo form should not be used as an indication of acceptable levels of intake of the racemic synthetic optical isomer.

AGRICULTURAL CHEMICALS

3324. γ-BHC on the brain

Solomon, L. M., West, D. P., Fitzloff, J. F. & Becker, A. M. (1977). Gamma benzene hexachloride in guinea-pig brain after topical application. *J. invest. Derm.* **68**, 310.

Gamma-benzene hexachloride (lindane; γ-BHC) undergoes significant absorption through the skin after topical application, individual variations in the degree of absorption being wide (*Cited in F.C.T.* 1975, **13**, 477). Its toxicity for the central nervous system (*ibid* 1969, **7**, 515) may be particularly significant, since, as the present study shows, the commercially available form of γ-BHC is concentrated in the brain tissue of guinea-pigs after topical application.

Newborn and 2-month-old guinea-pigs were given applications of 20 mg γ-BHC as a 1% commercial lotion. The lotion was applied uniformly to skin from which the hair had been closely clipped, and the amount of γ-BHC in blood and brain tissue 24 hr later was estimated by gas–liquid chromatography. Other animals were given a second application 24 hr after the first, and a third group received yet another application after a further 24 hr. Concentrations of γ-BHC in blood and brain increased with the number of applications of the lotion. In the newborns, blood concentrations rose from 0·43 to 0·81 μg/ml and brain concentrations from 4·5 to 15·6 μg/g after the first and third applications, respectively. In the 2-month-old animals, the corresponding figures were 0·12 and 0·57 μg/ml and 2·6 and 8·3 μg/g. Differences in the results for newborn and for adult guinea-pigs were not significant. Concentrations of γ-BHC in brain tissue were 12–28 times those in the blood.

3325. Infertility from dibromochloropropane

Whorton, D., Krauss, R. M., Marshall, S. & Milby, T. H. (1977). Infertility in male pesticide workers. *Lancet* **II**, 1259.

Testicular degeneration was the most striking pathological feature in rats exposed to 12 ppm atmospheric 1,2-dibromo-3-chloropropane (DBCP) for up to 92 days (Torkelson *et al. Toxic. appl. Pharmac.* 1961, **3**, 545). More recently, DBCP has been shown to induce stomach cancer in rats and mice (*Cited in F.C.T.* 1975, **13**, 144) and mutations in bacteria (*ibid* 1976, **14**, 505). Severe restrictions have been imposed in the United States on its handling and use, following the discovery of infertility in exposed workers (*Food Chemical News* 1977, **19** (26), 26). Details of these human reproductive effects have now been published.

In the agricultural chemical division of a Californian factory, where DBCP had been formulated since 1962, there were 25 non-vasectomized male employees. Sperm counts were found to be normal (≥40 million) in 11, between 10 and 30 million in three and not more than 1 million in the remaining 11. Lengths of exposure were strikingly related to sperm count, being below 3 months in the first group, 1–3 yr in the second and at least 3 yr (on average 8 yr) in the third. Only two of the last group were not azoospermic, and both of these displayed greatly reduced sperm motility and increases in abnormal forms. This group also demonstrated significant increases in serum levels of follicle-stimulating hormone and luteinizing hormone, but testosterone levels were unchanged. Testicular biopsies revealed loss of spermatogonia, with no evidence of inflammation or severe fibrosis.

Apart from their reduced sperm count the intermediate group appeared unaffected, as did the three female employees, and no other major abnormalities were detected in any group. DBCP levels measured in early 1977 were 0·4 ppm as a daily average, well above the TLV of 1 ppb now proposed (*Federal Register* 1978, **42**, 57266).

PROCESSING AND PACKAGING CONTAMINANTS

3326. Renal activity of lysinoalanine

Arnaud, M. J., Bracco, I., Magnenat, E. & Finot, P. A. (1977). Distribution of lysinoalanine by whole body autoradiography and cellular localization in the kidney, *Acta pharmac. tox.* **41**, 138.

Struthers, B. J., Dahlgren, R. R. & Hopkins, D. T. (1977). Biological effects of feeding graded levels of alkali treated soybean protein containing lysinoalanine (N^{ϵ}-2-(carboxyethyl-L-lysine) in Sprague-Dawley and Wistar rats. *J. Nutr.* **107**, 1190.

Lysinoalanine (LAL) occurs in protein foodstuffs after alkali treatment. When fed to weanling rats it has been found to induce nuclear enlargement and other changes in the renal tubular cells (Woodard *et al. Fd Cosmet. Toxicol.* 1977, **15**, 117), the nuclear enlargement being limited almost entirely to the outer edge of the medulla. The first paper cited above describes the administration of LAL labelled with L-[U-^{14}C]lysine to Sprague–Dawley rats by stomach tube or iv injection. Radioactivity was concentrated in the inner portion of the kidney cortex. A similar distribution was seen in the hamster and the mouse, but not in the quail. Autoradiographs from one rat given [4,5-^{3}H]lysine-labelled LAL by mouth and examined 24 hr later showed a correlation between the distribution of radioactivity in kidney sections and the location of the cells liable to develop karyomegaly in animals given LAL over a prolonged period.

The second paper explores the dose-response aspect of LAL and demonstrates a difference between the responses of Sprague–Dawley and Wistar rats. Weanling rats of both strains fed a dietary level of 30% alkali-treated soya-bean protein containing 1% LAL developed renal calcification, which was not seen in controls, but the effect was twice as severe in the Wistar rats. On the other hand, marked renal-cell cytomegaly was observed only in the Sprague–Dawley rats fed the 30% diet. The addition of LAL-containing protein to the diet at a level of 30% reduced feed efficiency and weight gain in both strains, but lower levels (5, 10 and 20%) did not. The dietary LAL-treated protein had no effect on blood urea nitrogen, creatinine or renal γ-glutamyl transpeptidase.

THE CHEMICAL ENVIRONMENT

3327. Where there's (tobacco) smoke there's cadmium

Østergaard, K. (1977). The concentration of cadmium in renal tissue from smokers and non-smokers. *Acta med. scand.* **202**, 193.

We made passing reference recently to the tendency for cadmium derived from cigarettes to accumulate in smokers with chronic obstructive lung disease (*Cited in F.C.T.* 1978, **16**, 289). The paper cited above looks further into the question of the increased intake and accumulation of cadmium by smokers.

The kidneys constitute the main site of cadmium accumulation in man, showing a gradual rise from birth, when they contain virtually none, to a peak at about 40–50 yr, when the level may exceed 2 mg/g renal-tissue ash. Concentrations of cadmium were determined, therefore, in autopsy tissues from a series of smokers and non-smokers. The 61 subjects studied were all aged between 45 and 65 yr and the records available included blood-pressure data as well as information on smoking habits.

Analysis of the renal tissues by atomic absorption spectrophotometry showed mean cadmium concentrations (expressed per g ash) of 953 μg in the 19 non-smokers and 1920 μg in the 42 smokers. The figure for smokers of pipes, cigars and cheroots (1494 μg/g) did not differ significantly from that for non-smokers but the concentrations in cigarette smokers were over twice as high as those in non-smokers, averaging about 2300 μg. This difference was apparent in the data for both normotensive and hypertensive individuals, although the levels in all the hypertensive patients were much lower than in the other groups (747 and 1643 μg/g in the hypertensive non-smokers and smokers, respectively, compared with 1269 and 2885 μg/g in the corresponding normotensive groups), a probable reflection of a skew distribution resulting from the apparent interrelation between renal cadmium and blood pressure.

On the basis of a number of other analytical studies, it has been estimated that cigarette smokers inhale 0·1–0·2 μg cadmium/cigarette. Absorption of cadmium from the lungs is assumed to be in the 25–50% range, giving an absorption of 0·5–2 μg from 20 cigarettes. The estimated daily absorption of cadmium from food (0·5–1·5 μg) may thus be approximately doubled by the smoking of 20 cigarettes/day.

3328. Hazards of cobalt and cigarette smoke

Wehner, A. P., Busch, R. H., Olson, R. J. & Craig, D. K. (1977). Chronic inhalation of cobalt oxide and cigarette smoke by hamsters. *Am. ind. Hyg. Ass. J.* **38**, 338.

The toxicity of cobalt as an industrial air pollutant is still controversial. Asthma and related allergic symptoms as well as progressive interstitial fibrosis of the lungs have been reported among tool makers engaged in milling heavy metals (*Cited in F.C.T.* 1972, **10**, 269). The metal dust in the working area contained up to 0·3% cobalt and 1% tungsten. Coates & Watson (*Ann. intern. Med.* 1971, **75**, 709) named cobalt as the probable offending agent, and experimental work in the miniature pig supported this view (*Cited in F.C.T.* 1976, **14**, 72). The above-cited study examines the chronic inhalation of cobalt oxide and cigarette smoke by hamsters in order to define more clearly the role of cobalt as a potential toxic and carcinogenic agent.

A group of 102 male Syrian golden hamsters received whole-body exposures to a respirable aerosol

of cobalt oxide (CoO), 7 hr/day, 5 days/wk for the duration of their natural lifespan. Half of these animals were also exposed to cigarette smoke for 10-min periods twice before and once after each dust exposure. A further group of 51 hamsters, which received daily three smoke exposures and a 7-hr sham dust exposure, served as smoke controls. Sham dust exposures consisted of keeping the animals under the exposure conditions for 7 hr/day without introducing the CoO aerosol. The final control group of 51 hamsters received daily three sham smoke exposures and one 7-hr sham dust exposure.

Emphysema was apparent in the lungs of the hamsters that died during the study following exposure to CoO. The severity of the emphysema increased with increasing survival time, leading to the formation of large confluent cavities formed from numerous ruptured alveoli. In addition, hyperplasia and hypertrophy of the alveolar lining cells became apparent, macrophages increased in number throughout the lungs, and lymphocytes and plasma cells infiltrated alveolar septa and accumulated in foci adjacent to bronchial and vessel walls. Hyperplasia of the epithelia of the bronchi and bronchioles was apparent early in the experiment and increased in severity, while cells lining the alveoli tended to become cuboidal and ciliated (alveolar bronchiolization). These changes were often focal and adjacent to small airways. Slight diffuse septal fibrosis was generally present in hamsters exposed to CoO for 15 months or longer. Development and the types of changes in the group exposed to CoO plus smoke were very similar to those in the group given CoO plus sham smoke exposures, except for a highly significant increase in laryngeal lesions among the smoke-exposed hamsters.

It was concluded that CoO exposure caused pneumoconiosis but did not affect either the incidence of other effects or the lifespan of the hamsters. The CoO did not exert any carcinogenic effect. In contrast, the cigarette smoke affected neither the incidence nor degree of pneumoconiosis but significantly increased the incidence of certain other lesions, including tumours, and reduced the mean body weights. Lifespans were significantly longer in the smoke-exposed groups.

3329. Lead and the human kidney

Inglis, J. A., Henderson, D. A. & Emmerson, B. T. (1978). The pathology and pathogenesis of chronic lead nephropathy occurring in Queensland. *J. Path.* **124**, 65.

Considerable effort has been spent in Queensland on identifying the renal lesions caused by lead in man. In this part of Australia, lead poisoning from lead-containing paint was common between 1890 and 1930 and a relatively high incidence of renal disease among young people in the same area, compared with that in the rest of the country, led to suspicions that some of this chronic renal disease might be a sequel to childhood lead poisoning. Several investigations designed to establish the type of renal injury caused by lead were therefore undertaken and the lead content of the bones of patients with renal lesions was determined.

The lesion prevalent in Queensland presented as a chronic nephritis, which could not be placed in any of the established categories of chronic renal disease. Among people who died of this type of unclassifiable renal lesion the mean lead content of the skull bones was 8 mg/100 g bone, whereas in those who died from chronic renal lesions of identifiable aetiology the mean lead content of the skull was 2 mg/100 g, a level similar to that in patients without any renal disease. The high lead levels in the skull bones provided a clear indication of excessive lead absorption in childhood.

The chronic renal lesion induced by lead was studied carefully in a series of 53 cases. These cases could be clearly differentiated into two groups according to the amount of fibrosis in the kidneys. Macroscopically, the kidneys showing moderate or severe fibrosis were severely but symmetrically contracted. The surface was granular without large scars, cysts were often visible and the pyramids were small but well preserved. The cortical tissue was diminished in these kidneys and the cortico-medullary demarcation was lost. The less fibrotic kidneys were also severely contracted and had a very thin cortex, but they were free from cysts and the cortico-medullary demarcation and cortical vascular markings were preserved. Microscopic examination showed that a large proportion of the glomeruli had disappeared without trace in both groups, but most extensively in those showing severe fibrosis. This finding was the most characteristic feature of the disease. Some of the remaining glomeruli were enlarged and a proportion were surrounded by fibrous tissue or showed signs of adhesions between the glomerular tuft and Bowman's capsule. The tubules were either dilated or atrophic. The dilated tubules conformed to the appearance associated with compensatory hypertrophy and were surrounded by small amounts of fibrous tissue. The atrophic tubules were in areas of dense fibrosis; they were also occasionally dilated and contained eosinophilic material. Moderate or severe fibrosis was accompanied by thickening of the interlobular and larger vessels.

The principal distinguishing features of this type of renal lesion were, therefore, the preservation of the pyramids and the complete disappearance of many of the glomeruli. The latter condition is only seen in cases of long-standing chronic glomerulonephritis.

[These renal changes may well provide the basis for the hypertension observed in man in some cases of chronic lead poisoning. The production of a degenerative renal lesion in man is in strong contrast to the situation in the rat. In that species, chronic administration of lead in fairly high doses induces a proliferative lesion which is generally accepted as neoplastic. Another major difference in the renal response of man and the rat to lead intoxication is that in man the prime target seems to be the glomerulus, whereas in the rat the renal tubules are the first structures affected, displaying inclusions in both cytoplasm and nucleus (*Cited in F.C.T.* 1973, **11**, 919).]

3330. Acrolein and the carcinogens

Feron, V. J. & Kruysse, A. (1977). Effects of exposure to acrolein vapor in hamsters simultaneously treated with benzo[*a*]pyrene or diethylnitrosamine. *J. Toxicol. envir. Hlth* **3**, 379.

Acrolein is widely used in industry. It is a highly reactive unsaturated aldehyde and has been shown to be present in relatively high concentrations (8·2 μg/40 ml puff) in the vapour-phase of cigarette smoke (Newsome *et al. Tob. Sci.* 1965, **9**, 102). The present study was undertaken because of the possible importance of saturated and unsaturated aldehydes in cigarette smoke for the genesis of pulmonary cancer. It has been speculated that if acrolein does play a part in the carcinogenic process induced by tobacco in mammals, its role may be mediated through action on nucleic acid polymerases (*Cited in F.C.T.* 1974, **12**, 578).

Groups of Syrian golden hamsters were evenly distributed within two inhalation chambers, one chamber for air exposure and the other for exposure to 4·0 ppm (9·2 mg/m^3) acrolein, 7 hr/day, 5 days/wk for a period of 52 wk. Four of the five groups of animals in each chamber were treated either with an intratracheal instillation of benzo[*a*]pyrene (BP) weekly at one of two dose levels (0·2 ml of either 0·175 or 0·35% BP in 0·9% NaCl), with an sc injection of 0·2 ml 0·0675% *N*-nitrosodiethylamine (NDEA) in 0·9% NaCl once every 3 wk, or with weekly sc injections of the vehicle, while the fifth group was given no treatment apart from the inhalation exposure. At the end of the 52-wk treatment period, three animals of each sex from each of the groups not treated with BP or NDEA were taken from each chamber, killed and extensively examined. The remaining hamsters were killed after 81 wk and examined only for changes in the respiratory tract.

Exposure to acrolein caused abnormal behaviour, slight growth retardation, slight increases in haemoglobin content and packed cell volume (in females), increases in relative lung weight (in females) and relative brain weight, a decrease in relative liver weight (in females), and rhinitis accompanied by hyper- and metaplasia of the epithelium in the nasal cavity. Respiratory tumours found in both males and females treated with BP or NDEA were of the type usually seen in hamsters following administration of these carcinogens.

There was no evidence of any carcinogenic activity due to acrolein. However, a co-carcinogenic effect of acrolein on BP carcinogenesis in the respiratory tract was indicated by (1) a slightly increased incidence of respiratory-tract tumours in acrolein-exposed males and females given the lower BP dose, compared with air-exposed animals of this BP group, (2) a slightly higher incidence and shorter latent period of respiratory-tract tumours in acrolein-exposed females given the higher BP dose than in air-exposed females given the same BP dose level, and (3) the occurrence of a few more malignant tumours of the respiratory tract after treatment with BP and acrolein than after BP treatment alone. However, in males given the higher dose of BP neither the incidence of respiratory-tract tumours nor their latent period was affected by acrolein exposure.

The enhancing effects of acrolein on BP carcinogenesis were therefore considered to be doubtful and inconsistent. Since, in addition, there were no significant differences in the incidence, site or type of NDEA-induced respiratory tumours between control and acrolein-exposed animals, it was concluded that acrolein could not be identified as a co-factor in respiratory-tract carcinogenesis.

3331. Crude effects in herring gulls

Miller, D. S., Peakall, D. B. & Kinter, W. B. (1978). Ingestion of crude oil: sublethal effects in herring gull chicks. *Science, N.Y.* **199**, 315.

The hazards of a liberal coating of crude oil on the surface of sea and shore birds are well known, as are the efforts that are made and the skills that have been developed to clean up at least some of the birds afflicted in this way and return them to their natural habitat. However, it appears that there is a relatively low rate of survival among birds that have been freed from oil and released, even among those that seem to be quite healthy. This suggests that a delayed toxic effect may be involved, and several studies have been undertaken, particularly in connection with the effect of crude oil on plasma osmoregulation. This process is clearly of vital importance to marine birds and can also be demonstrated in many coastal birds kept in a simulated marine environment. Basically, it is effected by excess salt absorbed from the intestine being excreted through the nasal glands.

Miller *et al.* (*loc. cit.*) found that herring-gull chicks maintained on sea-water and whole unsalted herring and given a single 0·2-ml oral dose of Kuwait or the heavier South Louisiana crude oil showed some impairment of plasma osmoregulatory capacity and cessation of growth, although they ate more food than controls achieving a daily weight gain of about 3%. When killed 8–9 days after dosing, the birds given the heavier crude showed marked pathological changes in the intestine, mainly oedema and cytoplasmic disruption of the epithelial cells, which contained many lipid droplets. Some evidence of similar but less severe effects was seen in the birds given Kuwait crude, but studies *in vitro* showed impairment of nutrient uptake in intestinal slices only from gulls given the heavier crude.

The effects on osmoregulation were seen in both treated groups, but were less severe in birds given the Kuwait oil. In both the intestine and nasal glands, the activities of the adenosine triphosphatase enzymes apparently involved in sodium and potassium transport were reduced by the oil treatment, and while this was compensated to some extent in the nasal gland by tissue hypertrophy, the findings paralleled the observed rises in plasma-sodium levels. The oil also caused hypertrophy of the adrenal gland and of hepatic tissue, the latter effect being accompanied by induction of the microsomal mixed-function oxidase system.

The dose used in this study was considered to be "environmentally realistic" and the findings suggest that while ingestion of such amounts (with contaminated food or water or during preening, for example) is not lethal, it may reduce a bird's capacity for long-term survival in the face of other stresses frequently encountered at sea.

3332. Fate of organotin in the body

Brown, R. A., Nazario, C. M., de Tirado, R. S., Castrillón, J. & Agard, E. T. (1977). A comparison of

the half-life of inorganic and organic tin in the mouse. *Envir. Res.* **13,** 56.

Increasing interest in the use of slow-release formulations of organotin compounds as antifoulants, molluscicides and schistosomiasis-control agents brings to the fore the question of the ecological impact of alkyltins and their long-term toxicity in animals. Previous studies in rats, principally on triethyltin, have demonstrated that some compounds of this type interfere with oxidative phosphorylation in rat-liver mitochondria (*Cited in F.C.T.* 1976, **14,** 648) and cause oedema of the white matter of the central nervous system (*ibid* 1978, **16,** 363). The relatively low toxicity of inorganic tin is attributable to its low level of absorption following oral administration and to its rapid turnover in the tissues (*ibid* 1975, **13,** 399). The extent of biological conversion of organotin to the inorganic form is thus, as with mercury, an important factor in the possible environmental impact of the proposed slow-release formulations on all levels of the ecosystem.

In a study of this question at the mammalian level, Swiss mice were given a single injection of 0·12 mg ^{113}Sn labelled bis(tributyltin) oxide (TBTO) or of ^{113}Sn-labelled stannous chloride (0·5 ml of a solution containing 10^{-8} g Sn/ml). Some of the latter group were given a further injection, 6 days later, containing 80 μg unlabelled stannous chloride. Whole-body radioactivity counts in the animals injected with ^{113}Sn were not affected by subsequent injection of unlabelled tin. Following the inorganic injection, the effective half-life of ^{113}Sn was found to be 25 days, and that of its decay product, indium-113m, 21 days. After injection of TBTO, the initial whole-body radioactivity decreased rapidly, but it slowed after about 10 days to a rate similar to that recorded after the inorganic ^{113}Sn injection.

The findings are interpreted as an indication that organotin may be converted into inorganic tin in the body. Alternatively, a small fraction of the dose of TBTO may become bound in an organ where its turnover is relatively slow. The clearance rate of Sn in either form does not appear to depend upon the quantity injected. From the effective half-life (the result of the combination of biological and physical decay) demonstrated in this study, the biological half-life of ^{113}Sn was calculated to be 29 days. Overall, the results suggest that the long-term accumulation of organotin is probably not a problem in mammals.

3333. The time factor in alkyltin irritancy

Middleton, M. C. & Pratt, I. (1977). Skin water content as a quantitative index of the vascular and histologic changes produced in rat skin by di-*n*-butyltin and tri-*n*-butyltin. *J. invest. Derm.* **68,** 379.

Another problem that has arisen in connection with alkyltin compounds is their irritant effect on the skin and other tissues. A dibutyltin dilaurate stabilizer present in PVC implants caused severe tissue reactions in 2–4 days in the subcutaneous tissues of the rat and in rabbit muscle (*Cited in F.C.T.* 1966, **4,** 117) and both di-*n*-butyltin (DBT) and tri-*n*-butyltin (TBT) are known irritants of human and animal skin (Barnes & Stoner. *Br. J. ind. Med.* 1958, **15.** 15; Lyle, *ibid* 1958, **15,** 193). Three vascular changes produced in rat skin by these compounds were therefore studied in a search for a representative and quantitative measure of the overall vascular response.

Applications of 67 nmol DBT/cm^2 to the dorsal skin of rats produced slight local changes including minor increases in vascular permeability and in the water content of the tissue and some erythema. The same dose of TBT caused greater changes, including cellular necrosis, marked increases in vascular permeability and tissue water and a more rapid and severe development of erythema. Similar relatively severe changes were seen after application of 335 nmol DBT/cm^2. Maximal increases in all three parameters (vascular permeability, oedema and erythema) were seen 2–3 hr after application of 67 nmol TBT/cm^2, but with 335 nmol DBT/cm^2 permeability reached its peak at 3 hr and erythema at 12 hr, while oedema was high between 12 and 48 hr. The latter findings suggest that these three effects are not directly related. The more rapid responses to TBT probably reflected the compound's penetration of the skin *via* the hair follicles, around which the earliest damage was localized.

In spite of the differences in timing, each of the three vascular parameters gave the same ranking order for the three test solutions. The change in the water content of the skin is the parameter most useful for quantitative assessment, however, and provided a time course of the response is determined, it may provide a useful measure of the vascular changes and the corresponding cellular injury produced by skin irritants.

3334. 4,4′-Diaminodiphenyl ether induces liver tumours

Steinhoff, D. (1977). Cancerogene Wirkung von 4,4′-Diamino-diphenyläther bei Ratten. *Naturwissenschaften* **64,** 394.

Rats injected sc with 3,3′-dichloro-4,4′-diaminodiphenyl ether have been shown to develop multiple lesions of the epithelium of the auditory canal and deep ceruminal gland, lesions that may progress into papillary and squamous-cell carcinomas (*Cited in F.C.T.* 1975, **13,** 589). The related 4,4′-diaminodiphenyl ether (DADPE) has demonstrated nephrotoxicity and hepatotoxicity after oral or subcutaneous administration, and has been suspected of weak carcinogenicity without any identification of a specific target organ. Further data regarding its carcinogenic capacity are now reported.

Chemically pure DADPE was injected sc in saline solution into Wistar rats in the maximum acceptable dose (100–300 mg/kg) once weekly. By day 670, several tumours had appeared. Treated rats, given a total dose of 14·4 g DADPE/kg, had a mean lifespan of 860 days, compared with 907 days in saline-injected controls. The incidence of death from malignant tumours was 55% in treated rats and 26% in controls, the corresponding figures for benign tumours being 55 and 20%. Malignant liver tumours, multiple in three of the 40 animals, appeared in 25% of the treated group, and benign liver tumours, multiple in 11 cases, occurred in 30%; no liver tumours were found in the control rats. The hepatotoxicity of DADPE was also demonstrated in the other treated rats, in which evidence of liver necrosis and cirrhosis was found.

3335. Mutagenic epichlorohydrin

Kučerová, M., Polívková, Z., Šrám, R. & Matoušek, V. (1976). Mutagenic effect of epichlorohydrin. I. Testing on human lymphocytes *in vitro* in comparison with TEPA. *Mutation Res.* **34,** 271.

Šrám, R. J., Černá, M. & Kučerová, M. (1976). The genetic risk of epichlorhydrin as related to the occupational exposure. *Biol. Zbl.* **95,** 451.

Kučerová, M., Zhurkov, V. S., Polívková, Z. & Ivanova, J. E. (1977). Mutagenic effect of epichlorohydrin. II. Analysis of chromosomal aberrations in lymphocytes of persons occupationally exposed to epichlorohydrin. *Mutation Res.* **48,** 355.

Epichlorohydrin (ECHH) is widely used as a solvent for resins, gums, cellulose esters, paints and lacquers and as a raw material in the manufacture of epoxy resins (see below). Mutagenic effects of ECHH have been described in a number of organisms, including *Neurospora crassa* (Westergard, *Experientia* 1957, **13,** 224) and *Escherichia coli* (Strauss & Okubo, *J. Bact.* 1960, **79,** 464), although Epstein *et al.* (*Toxic. appl. Pharmac.* 1972, **23,** 288) obtained negative results in the mouse dominant lethal assay. Because of the occupational risks involved, Kučerová and her co-workers investigated the possibility of mutagenic effects of ECHH on human chromosomes. Their study was divided into two parts covering the mutagenic effects of ECHH on human lymphocytes *in vitro* and observations on the peripheral lymphocytes of occupationally exposed workers. In addition, workers of the same group made a separate study using a battery of mutagenicity tests.

The effects of ECHH on human lymphocytes *in vitro* were compared with those of tris-(1-aziridinyl)-phosphine oxide (TEPA), which has previously been demonstrated to be a strong mutagen. Solutions of TEPA and ECHH were added to 56-hr cultures of human lymphocytes for the last 24 hr. ECHH was added in 0·9% saline–DMSO (1:1) to give final concentrations of 10–0·00001 μM in the medium, the same concentrations being used for TEPA. Controls were used both with and without DMSO. A significant dose-dependent increase in aberrations was evident after exposure to either compound, but TEPA was much more powerful than ECHH, particularly at high concentrations. Of the four types of aberration observed, chromatid and chromosomal breaks were commonest, while chromatid exchanges were rare and chromosomal exchanges were extremely rare. These results were in agreement with previously published data on ECHH. This study also entailed an investigation of the effects of adding the mutagen to the culture medium at different stages. The ECHH or TEPA, in the most effective concentrations, was added to the medium either for 1 hr at the start of cultivation, for 1 hr between the 24th and 25th hr of cultivation or for the last 24 hr of cultivation. It was found that in the first two cases (involving exposure of cells only in the G_0 or the G_1 and early S phases) only insignificant increases in the number of impaired chromosomes occurred, whereas when either of the mutagens was added for the last 24 hr, there were very significant increases in the number of cells affected.

In a separate study by workers of the same group, a whole battery of tests was applied to ECHH. Mutagenic activity of ECHH was demonstrated in *Salmonella typhimurium* using strains G46 and TA100 without metabolic activation. ECHH increased the frequency of back mutations in a host-mediated assay with *S. typhimurium* and chromosomal aberrations were detected by mouse bone-marrow analysis as well as in human peripheral lymphocytes exposed *in vitro*. A dose-effect relationship was observed particularly in the mouse bone-marrow tests. However, in agreement with the findings of Epstein *et al.* (*loc. cit.*) no changes were found in a dominant lethal test. The results of the mouse bone-marrow test were used to estimate the risk to workers occupationally exposed to an average of 0·5 mg/m^3 for 6-hr periods. These calculations predicted an increase in aberrations to 2–2·5 times the control rate in the course of 1 yr, a rise considered by the authors to be below the maximum admissible risk.

This estimate was considerably above the increase actually found by the group in a further study focusing on chromosomal changes in the peripheral lymphocytes of occupationally exposed workers. Blood samples from 35 workers (aged 23–54 yr) were taken at three stages. The first samples (control) were taken just before work started at a new chemical plant producing ECHH. The second and third samples were taken after exposure for 1 and 2 yr respectively. Workers were exposed to high doses of ECHH, 0·5–5·0 mg/m^3, averaging above the Czechoslovakian maximum acceptable concentration but well below the TLV of 20 mg/m^3. The percentage of aberrant peripheral lymphocytes found in the first sample was 1·37, a level previously found to occur spontaneously. After 1 yr the frequency rose significantly to 1·91% and a further statistically significant increase to 2·69% had occurred by the end of yr 2. In common with the *in vitro* study, the changes were mainly chromatid and chromosomal breaks, rather than exchanges. Although there has been criticism of this method as a means of testing weak mutagens, the authors believe that it is reliable when the correct techniques are used with adequate controls, and when the subjects "are not concurrently exposed to a combination of other suspected or known mutagens".

3336. Epoxy resin sensitization versus molecular weight

Thorgeirsson, A. & Fregert, S. (1977). Allergenicity of epoxy resins in the guinea pig. *Acta derm.-vener., Stockh.* **57,** 253.

Epoxy resins and their hardeners rank among the sensitizers most commonly responsible for cases of contact allergy (*Cited in F.C.T.* 1974, **12,** 582), but it seems that little information is available on the significance of the molecular weight of these resins in relation to their sensitizing capacity. The most common type of epoxy resin, the diglycidyl ether–Bisphenol A type, is a reaction product of epichlorohydrin and 2,2-bis-(*p*-hydroxyphenyl)propane. The molecular weight can therefore vary between 340 and more than 10,000, molecular weights below 908 giving liquid or semi-solid resins. The marketed resins, such as those used with one exception in this study, are

mixtures, identified by average molecular weight (mol wt) but not by the distribution of resins of differing molecular size.

The study used the well-known guinea-pig maximization test (Magnusson & Kligman, *J. invest. Derm.* 1969, **52,** 268) comprising intradermal injection followed after 1 wk by topical application and after a further 2 wk by a 24-hr occluded-patch test. Equimolar concentrations of six resins of the diglycidyl ether–Bisphenol A type (mol wt 340–1850) dissolved in acetone (or methyl ethyl ketone for the mol wt-1850 sample) were tested on groups of 20 animals.

The isolated resin of lowest possible molecular weight (340) and the mixed resin of mol wt 350 sensitized all 20 guinea-pigs tested, while with rising mol wt of the resin the numbers sensitized per group decreased to 17 (mol wt 480), 11 (900), 6 (1280) and 0 (1850). Bisphenol A alone failed to induce sensitization in 15 guinea-pigs, but epichlorohydrin alone sensitized 9 of 15. Sensitization to a resin did not involve cross sensitization to bisphenol A or epichlorohydrin. Cross-sensitization to the three resins of lower mol wt was observed in most animals, irrespective of the resin used for sensitization, but the cross-sensitization rate was low with the higher resins.

This suggests that the sensitization capacity may be a property only of the individual compounds of lowest molecular weight, which could be present, for example, in the mol wt-1850 resin in sufficient quantity to induce sensitivity on intradermal injection but not to elicit a reaction to epicutaneous challenge. Further study of this hypothesis must await the isolation of other individual resins, only that of molecular weight 340 being available so far.

3337. The fish and the flame-retardant

Maylin, G. A., Henion, J. D., Hicks, L. J., Leibovitz, L., Ahrens, V. D., Gilbert, M. & Lisk, D. J. (1977). Toxicity to fish of flame retardant fabrics immersed in their water. Part I. *Bull. env. contam. & Toxicol.* (*U.S.*) **17,** 499.

Eldefrawi, A. T., Mansour, N. A., Brattsten, L. B., Ahrens, V. D. & Lisk, D. J. (1977). Further toxicologic studies with commercial and candidate flame retardant chemicals. Part II. *Bull. env. contam. & Toxicol.* (*U.S.*) **17,** 720.

In no consumer-product area is the conflict between risk and benefit more immediately apparent than in the development of flame-retarding treatments for fabrics, and few would envy the authorities the task of weighing the possibility of reducing the severe or fatal burning of children, for example, against the less clearly defined possibility that those children might, in the long term, be affected adversely as a result of absorbing through their skin the chemicals used to make their nightwear flame-proof. Effects of these chemicals on other sectors of the environment have also to be considered. Details of extensive toxicity studies undertaken on one type of flame-retardant are reviewed elsewhere in this issue (p. 492) and some time ago we noted a report that certain organophosphorus compounds, which could be leached from treated fabrics during laundering, were toxic to goldfish at concentrations of 1 ppm (*ibid* 1976, **14,** 512). Further studies now serve to fill in some of the details of this particular hazard.

In the first paper cited above, samples of fabrics treated with mixed cyclic phosphonates (Antiblaze 19), an oligomeric vinyl phosphonate (Fyrol 76), *N*-methyloldimethyl phosphonopropionamide (Pyrovatex CP), tris-(2,3-dibromopropyl) phosphate (TDBPP) or tetrakis-(hydroxymethyl)phosphonium hydroxide (THPOH) were immersed in water containing goldfish (a 7 in. × 15 in. sample in 20 litres water containing six fish). TDBPP consistently killed the fish within 24–168 hr, death being preceded by hyperactivity, colour fading, anorexia, bulging of eyes, head and body, extension of scales, disorientation, sluggishness, rapid gill movement and air-seeking. The maintenance of fish in water to which 1 ppm TDBPP had been added induced similar effects, some of which were characteristic of anticholinesterase intoxication. Histopathological examination showed subcutaneous and intramuscular oedema, with focal necrosis in liver and kidney. TDBPP-finished fabrics that had been laundered affected the fish less rapidly when immersed in the water. The toxicity of TDBPP is not due to its hydrolysis to 2,3-dibromopropanol, since exposure of goldfish to 1 ppm of this compound for 7 days had no observable effect. Analysis of the water in which they were immersed indicated that, when unlaundered, the treated polyester fabric released 75–140 μg TDBPP/in^2 in 24 hr. The retardant subsequently lost HBr and may have been hydrolysed or photolytically degraded to 2-bromo-2-propenyl-bis-(2,3-dibromopropyl) phosphate, the possible piscicide. The predominantly cellulosic fabrics carrying the other flame-retardant chemicals were not toxic to the fish over the 7-day immersion period, probably because of chemical bonding between the agent and the fabric.

The second paper cited reports further on the results of adding flame-retarding agents directly to water containing goldfish. The compounds tested were Antiblaze 19, monomeric chloroethyl phosphonates with high-boiling phosphonates (Antiblaze 78), tris-(β-chloroethyl) phosphate (Fyrol CEF), tris-(dichloropropyl) phosphate (Fyrol FR-2), Fyrol 76, tri-(*o*-cresol) phosphate (Phosflex 179-C), mixtures of triaryl phosphate esters containing halogen (Phosflex 300 and Phosflex 400), Pyrovatex CP, TDBPP and THPOH. Signs of toxicity and deaths among the fish were noted as a function of time. Notable toxicity was seen with Fyrol FR-2, Phosflex 300 and 400, TDBPP and THPOH, with sluggish and disorientated swimming preceding death. In tests using the electric organ of *Torpedo ocellata*, the electric ray, these compounds and Fyrol CEF showed some acetylcholinesterase-inhibiting capacity, and in microsomal preparations from the armyworm (*Spodoptera eridania*), THPOH almost totally inhibited *p*-chloro-*N*-methylaniline *N*-demethylase; Fyrol CEF and FR-2, and TDBPP were fairly potent and Fyrol 76 and Pyrovatex CF were almost inactive in this respect. Ingestion of the agents by houseflies caused few deaths, except when the synergist piperonyl butoxide was also added to the food.

NATURAL PRODUCTS

3338. More on congeners and alcohol metabolism

Auty, R. M. & Branch, R. A. (1977): Pharmacokinetics and pharmacodynamics of ethanol, whiskey, and ethanol with n-propyl, n-butyl, and iso-amyl alcohols. *Clin. Pharmac. Ther.* **22**, 242.

Congeners present in alcoholic beverages may affect ethanol elimination, but the significance of any such effect for the moderate drinker is uncertain (*Cited in F.C.T.* 1977, **15**, 653). A study of the influence of the three predominant congeners, *n*-propanol, *n*-butanol and isoamyl alcohol, on the pharmacokinetics of ethanol in six normal men, all moderate drinkers, throws some light on this problem.

In a randomized double-blind, crossover trial, each drink (ethanol alone, whiskey or a mixture of ethanol with the three higher alcohols) was equivalent to 1 g ethanol/kg body weight, diluted to 600 ml with orange juice and consumed at a constant rate over 20 min. Blood samples were taken and reaction times and electroencephalograms (EEGs) were recorded before the dose, at 20-min intervals after the dose for 2 hr and then at 30-min intervals for 3 hr. All three drinks produced similar plasma-ethanol concentrations eventually, but the peak was reached more slowly after whiskey ingestion (mean 83 min) than after ethanol alone (63 min), and was even more delayed (97 min) after the ethanol–higher alcohol mixture, in which the concentration of the congeners was about twice that in the whiskey. The terminal phase of ethanol elimination was consistent with exponential decay in all the studies, the half-life being longer after the ethanol-higher alcohol mixture than after ethanol or whiskey. Reaction time was prolonged by each treatment, reaching a peak after about 2 hr and returning to normal within 5 hr, but deterioration in performance 2 hr after whiskey was less than that after the ethanol–higher alcohol mixture than after ethanol or whiskey. Reaction time was prolonged by anol concentrations when data for the full 5-hr period after alcohol administration were considered, the increase in reaction time correlated positively with plasma-ethanol concentration during the linear phase of ethanol elimination after ethanol alone and after the ethanol–higher alcohol mixture. Alpha activity (8–14 Hz) in the EEG was reduced by all drinks, with a maximum fall in mean dominant frequency 2·5 hr after the start of drinking. Subjects could not distinguish between the three beverages, and their reports of the severity of effects were unrelated to the type of drink consumed. There was no evidence suggesting that whiskey has unique effects not produced by ethanol alone.

3339. Intestinal absorption of oxalate

Madorsky, M. L. & Finlayson, B. (1977). Oxalate absorption from intestinal segments of rats. *Investve Urol.* **14**, 274.

Oxalate is ingested in many foods, particularly spinach, rhubarb, parsley and tea, and its excretion, together with that of calcium, is important in relation to the formation of calculi in the kidney and urinary bladder (*Cited in F.C.T.* 1969, **7**, 395). Accordingly the rate at which dietary oxalate is absorbed, to augment the body burden of endogenous oxalic acid (see below), is also significant. Isolated intestinal segments of the jejunum, ileum and colon of rats were examined at intervals from 5 to 60 min after intraluminal injection of ^{14}C-labelled oxalate solution in saline. The initial absorption of oxalate was rapid, at 6·5%/min, but decreased after 5 min to about 0·6%/min. Absorption was greatest in the jejunum and least in the colon. A rapid initial influx of water into the gut lumen may have been a response to the "noxious effect" of oxalate on tissue, since the experimental challenge solutions were nearly iso-osmolar with plasma. The abrupt reduction of oxalate absorption after about 5 min suggests that oxalate may inhibit its own absorption.

Oxalate absorption was also studied with solutions of [^{14}C]oxalate containing a 4 mM concentration of calcium chloride, magnesium chloride or sodium oxalate. No difference in the apparent rate of [^{14}C]oxalate transfer was produced by addition of these salts, except in the jejunum, where absorption was accelerated by magnesium chloride. It is calculated that at least 8·6% of a challenge dose of oxalate should be absorbed from the small bowel during normal food transit. Since the addition of magnesium failed to decrease [^{14}C]oxalate absorption in the rat model, it is unlikely that supplementation of the diet with magnesium in attempts to alleviate or prevent the formation of calcium oxalate calculi can be justified on the grounds that it reduces oxalate absorption; any effective contribution of magnesium to the treatment of oxalate stone disease in man seems to require some other explanation.

3340. Vitamin C and oxalate stones

Du Bruyn, D. B., De Klerk, W. A. & Liebenberg, N.v.d.W. (1977). High dietary ascorbic acid levels and oxalate crystallization in soft tissues of baboons. *S. Afr. med. J.* **52**, 861.

Oxalic acid is one of the end-products of ascorbic acid (AA) metabolism, and this has led to fears that the ingestion of massive amounts of this vitamin could lead to the formation of kidney or bladder stones. In the rats given large daily injections of AA there was a sevenfold increase in the weight of calculi formed round a lead ball inserted in the bladder, and the amount of oxalic acid in the deposit was more than doubled (*Cited in F.C.T.* 1967, **5**, 111). In man, a single dose of 6 g AA produced a significant increase in the urinary content of microcrystals 60 μm or more in diameter (von Sengbusch & Timmermann, *Urol. int.* 1957, **5**, 218). There have also been brief reports of two men who passed urinary stones after taking either 2 g AA daily for 2 wk (Briggs, *Lancet* 1973, **ii**, 1439) or 1 g daily for several months (Roth & Breitenfield, *J. Am. med. Ass.* 1977, **237**, 768), oxalate excretion in the latter case being approximately halved when AA supplements were withdrawn.

Captive baboons require considerable AA supple-

mentation (about 20 mg/kg/day) to maintain the serum-AA levels of 1·0–1·5 mg/100 ml found in free-living animals, and they were therefore chosen for a study of possible oxalate-crystal formation from AA. Semi-synthetic diets containing 200 or 2500 mg AA/100 g dry ration, equivalent to a daily dose levels of about 80 or 1000 mg/kg, respectively, were fed for 20 months. These diets produced average serum-AA levels of 1·76 and 2·2 mg/100 ml, respectively. The baboons ate well and remained in excellent condition throughout, and on autopsy there was nc gross or microscopic evidence of oxalate crystals in the kidney, bladder or other organs examined. Calcium oxalate crystals found in the kidneys of a female free-living baboon (McConnell *et al. Onderstepoort J. vet. Res.* 1974, **41**, 97) were therefore thought likely to have originated from oxalic acid present in plants eaten in the wild.

3341. Food allergy

Finn, R. & Cohen, H. N. (1978). "Food allergy": fact or fiction? *Lancet* **I**, 426.

Disorders such as skin rashes or gastro-intestinal problems, caused by altered immunological mechanisms, are accepted as one of the consequences of food allergy. Some authors claim that these are not the only effects of an 'altered reactivity' to food, and that complaints such as headache, palpitations, vomiting, panic attacks and anxiety may also be caused by particular constituents of the diet. This broader concept of 'food allergy' is not widely accepted within the medical profession, presumably because objective evidence is difficult to obtain.

Six case reports of patients with clearly documented evidence of signs and symptoms related to the intake of specific items of food have been recorded in the paper cited above. Three of these had objective signs of disease. One (case 1) complained of palpitations and dyspnoea, another (case 3) had had large and painful aphthous ulcers since infancy and case 5 had suffered from nausea and vomiting since childhood. Case 1 regularly drank vast quantities of tea, and during clinical investigations gastric intubation of tea, coffee or tomato juice, but not of water, provoked an acceleration of the heart rate. Intragastric administration of tea on five occasions provoked a bout of vomiting each time in case 5, while in case 3 withdrawal of potato, coffee and chocolate from the diet led to a complete healing of the ulcers. Enquiries revealed the rather strange fact that for most of her life this patient had eaten raw potato almost daily!

Case 2 had complained of severe headaches over many years, but thorough neurological investigations were negative. He was, however, a heavy coffee drinker (more than 20 cups/day) and when he stopped taking coffee, his headaches and other symptoms disappeared. In case 4 tea was responsible for severe recurrent pain, simulating renal colic, in the right side of the abdomen. An acceleration of the heart rate and abdominal pain occurred within 24 hr of each of five intragastric administrations of tea but not after water, and after he stopped drinking tea the pain did not recur. Alcohol ingestion appeared to have a similar effect in this patient. Case 6 developed symptoms of panic, lethargy, depression and faintness in the course of treatment for severe hypertension. His high blood pressure was found to be due to renal-artery stenosis and when this was treated surgically the hypertension was cured, but his other symptoms remained. It was found that he was a heavy coffee and tea drinker and when this habit was broken his mental problems disappeared.

[In the past, food additives have often been blamed for skin or gastro-intestinal disturbances, accompanied in some instances by effects not unlike some of those mentioned above. It seems, however, that it is as important to look carefully into the types of food and drink ingested by people with such complaints as it is to investigate their encounters with specific food additives, since the former may be as likely as the latter to be implicated in the problem.]

3342. Inhibition of bracken carcinogenesis

Pamukcu, A. M., Yalçiner, S. & Bryan, G. T. (1977). Inhibition of carcinogenic effect of bracken fern (*Pteridium aquilinum*) by various chemicals. *Cancer, N.Y.* **40**, 2450.

Bracken is frequently among the plant population of all but the best of pasture-lands. Unfortunately it must carry the can for a high incidence of bladder tumours in cattle (*Cited in F.C.T.* 1970, **8**, 85) and of both intestinal and bladder tumours in sheep (*ibid* 1966, **4**, 358; Harburrt & Leaver, *Aust. vet. J.* 1969, **45**, 473). Previous work has attempted to trace the carcinogenic components of bracken; shikimic acid produces neoplasms of the gastric mucosa in mice (*Cited in F.C.T.* 1975, **13**, 405) and incorporation of tannin in the cholesterol increases the incidence of bladder tumours in rats implanted with cholesterol pellets (*ibid* 1976, **14**, 651). Attention seems now to be turning to the possible inhibitors of bracken carcinogenesis.

Pamukcu *et al.* (cited above) tested the effects of ten different dietary regimes on tumour formation in rats. Groups 1–5 were given basic diet and dried bracken in the ratio 2:1, with the addition (per gram of diet) of 5 mg butylated hydroxyanisole (BHA; group 2), 5 mg disulfiram (group 3), 20 mg calcium chloride ($CaCl_2$; group 4) or 50 mg cross-linked polyvinylpyrrolidone (PVP; group 5). Groups 6–10 followed the same pattern, but in all cases the mixture of basic diet with bracken fern was replaced by basic diet alone. There were no intestinal or bladder tumours in any of the control groups not receiving bracken, in sharp contrast to animals in groups 1–5. In group 1, 100% of rats had intestinal tumours but this was reduced significantly by BHA, disulfiram and $CaCl_2$, although not PVP. Urinary bladder tumours were present in 73% of rats given basic diet with bracken. This percentage was reduced to 17·4 or 18%, respectively, in animals given $CaCl_2$ or PVP, although no significant change was brought about by either BHA or disulfiram. Thus PVP inhibits bracken-induced carcinogenesis in the bladder but not the intestine, whereas $CaCl_2$ acts at both sites. Previous studies, such as that by Dollahite & Camp (*Am. J. vet. Res.* 1962, **23**, 1271), have indicated that both

compounds have the capacity to absorb or precipitate tannin and other flavonoid compounds present in bracken. Another possibility is that PVP and $CaCl_2$ form insoluble complexes with the carcinogenic substance in bracken and inhibit its absorption. BHA and disulfiram both suppress the capacity of bracken to induce tumours in the intestine but not in the bladder. The mechanism of this inhibitory action is not known but it seems possible that it is similar for the two compounds. The antioxidant properties of BHA, in particular, may be significant, as may be the capacity of disulfiram to inhibit certain enzymes.

COSMETICS, TOILETRIES AND HOUSEHOLD PRODUCTS

3343. Skin penetration by another pyrithione

Wedig, J. H., Feldmann, R. J. & Maibach, H. I. (1977). Percutaneous penetration of the magnesium sulfate adduct of dipyrithione in man. *Toxic. appl. Pharmac.* **41**, 1.

The broad antimicrobial and antifungal spectrum of pyridine-2-thiol-1-oxide (pyrithione) is valuable to microbiologists and cosmetic chemists. The percutaneous absorption of sodium pyrithione has been shown to be low in rats and monkeys (*Cited in F.C.T.* 1970, **8**, 147) and dermal toxicity studies on zinc pyrithione have shown no adverse effects in rats and rabbits (*ibid* 1966, **4**, 554; *ibid* 1976, **14**, 366). The magnesium sulphate adduct of 2,2'-dithiobis-(pyridine-1-oxide) (MDS) also has antimicrobial properties, as well as being usefully soluble in polar and non-polar solvents, and its percutaneous penetration has now been studied.

Urinary excretion of MDS after application of a methanolic solution providing 4, 12 or 40 $\mu g/cm^2$ to an area of about 11·4 cm^2 on the forearm, forehead or scalp of adult volunteers was used as a measure of percutaneous absorption. The application was allowed to remain for 8 hr, and urine samples were taken for 5 days. MDS labelled with ^{14}C was used as marker. Urinary excretion was corrected on the basis of measurements of the urinary recovery of small iv doses of MDS.

No untoward effects were observed in any subjects receiving an iv or dermal dose of [^{14}C]MDS. After iv injection, the excretion of MDS in urine followed a biphasic exponential course indicating some tissue binding or plasma storage, and enterohepatic circulation was indicated by the appearance of radioactivity in the faeces. After dermal application there was no indication that MDS was subject to enterohepatic cycling. Skin penetration of MDS from all test areas tended to increase with increasing concentration of the solution, and was greater for the forehead and scalp than for the forearm. The percentages of the dose excreted in the urine over the first 120 hr after treatment were 2·7–3·2% for the forearm, 11·1–15·3% for the forehead and 6·2–13·2% for the scalp. Although not directly comparable, these results are of the same order as the earlier results with sodium pyrithione in rats and monkeys.

3344. The light shines on bergamot

Zaynoun, S. T., Johnson, B. E. & Frain-Bell, W. (1977). A study of oil of bergamot and its importance as a phototoxic agent. I. Characterization and quantification of the photoactive component. *Br. J. Derm.* **96**, 475.

The phototoxic effect of oil of bergamot (*Citrus bergamia*), which is a frequent component of perfumes, has been ascribed to the presence of the furocoumarin bergapten (5-methoxypsoralen; 5-MOP) at a concentration greater than 0·001% (*Cited in F.C.T.* 1971, **9**, 604).

Further testing of various samples of bergamot oil for phototoxicity, using *Candida albicans* as the test organism and by patch-testing on normal volunteers, has involved a comparison with solutions of pure 5-MOP and xanthotoxin (8-methoxypsoralen; 8-MOP). Three samples of bergamot oil were assayed for 5-MOP by thin-layer chromatography (TLC) followed by solvent extraction and measurement of the absorbance at 310 nm. Two samples contained 0·31 and 0·29 g 5-MOP/100 g while the third contained no 5-MOP at all.

TLC fractions were plated on Sabouraud dextrose-agar seeded with *C. albicans* and exposed to 'black light', and were also applied in open photopatch tests on fair-skinned volunteers. Similar patch tests were carried out on ethanolic solutions of the bergamot oil containing 0·31 g 5-MOP/100 g and with various ethanolic dilutions of pure 5-MOP and 8-MOP for comparison. The sample of oil that contained no detectable 5-MOP proved to be inactive in photopatch tests and, of the TLC fractions of the other two samples, only the 5-MOP fractions inhibited growth of *C. albicans* and gave a positive result in photopatch tests. In the tests on the bergamot oil containing 5-MOP and on pure 5-MOP in ethanol, the level of positive photopatch test results correlated with the concentration of 5-MOP in the solutions. A 63% positive level of photopatch results was produced by solutions of 2·5 mg pure 5-MOP/100 ml and of 1·0 ml of the active bergamot oil sample/100 ml (the latter solution containing 2·7 mg 5-MOP/100 ml). These results demonstrate the importance of using a bergamot oil of known 5-MOP content for photopatch testing to ensure the presence of the active component and avoid false negative results. The findings also support previous indications that 5-MOP is the only significant phototoxic agent in bergamot oil, but in contrast to some other reports, pure 8-MOP was only slightly more active than 5-MOP in these experiments on human skin.

CANCER RESEARCH

3345. Difficulties with the bladder

Brill, E. (1977). The role of dog bladder mucosa in the *N*-oxidation of arylamines. *Res. Commun. chem. Path. Pharmac.* **16,** 73.

The bladders of the dog and man show some similarity in their susceptibility to a number of carcinogens, notably 2-naphthylamine and 4-diphenylamine. Whilst the biochemical interaction between arylamines and the bladder of the dog is therefore obviously worthy of study, some of the difficulties this presents are illustrated by the present investigation, involving 2-naphthylamine and 4-diphenylamine, which are bladder carcinogens in the dog, 2-fluorenylamine and 3-dibenzofuranylamine, which are bladder carcinogens in the rat, and 1-naphthylamine, which shows only very weak, if any, carcinogenic activity in the dog bladder.

Each of these compounds, in 1 μM concentration, was incubated with an intact bladder of a dog or with isolated bladder mucosa. An attempt was made to measure the extent of their oxidation to the *N*-hydroxylated derivative both by gas chromatography and indirectly using the ability of the *N*-hydroxyarylamine to oxidize the haemoglobin of dog erythrocytes. Control experiments demonstrated the feasibility of the latter method: up to 48% oxidation of the haemoglobin occurred in incubation mixtures in which the arylamine was replaced by 0·1 μM-*N*-hydroxyarylamine. On the evidence of ferrihaemoglobin production, the intact bladder induced only a very limited degree of hydroxylation of any arylamine; the isolated mucosa exhibited even lower activity, typically about half that of the intact bladder.

Oxidation of the arylamine could not be confirmed by gas chromatography. Moreover, the concentration of *N*-hydroxyarylamine in the control experiments was shown to decrease significantly with incubation time, suggesting that it was either decomposing or binding irreversibly to the bladder mucosa. The superiority of an 'erythrocyte' detection system was therefore presumably due to the continual reaction of the newly formed hydroxylated derivative with haemoglobin.

In order to overcome the apparently low activity of the limited amount of tissue obtained from a single bladder, each arylamine was incubated with microsomes from the mucosa obtained from a large number of dog bladders. The microsomes were isolated after the bladder tissues had been first minced and then powdered under liquid nitrogen, an experimental technique which, when applied to dog livers, produced a metabolically active microsomal fraction. Although control experiments showed that both the haemoglobin-oxidation and chromatographic methods were capable of detecting *N*-hydroxyarylamine, no oxidation product could be detected in any incubation mixture containing dog-bladder microsomes.

N-Hydroxyarylamines have been detected in the urine of dogs receiving arylamines orally, but the results of this study suggest that the bladder plays only a minor role in the formation of these presumed proximate carcinogens. Unpublished *in vitro* studies by the author cited above are said to show that the liver may be the organ mainly responsible for the activation of carcinogenic arylamines in the dog.

3346. Bedding down on cedar

Vlahakis, G. (1977). Possible carcinogenic effects of cedar shavings in bedding of C3H-A^{vy}fB mice. *J. natn. Cancer Inst.* **58,** 149.

A high incidence of mammary and liver cancer in some batches of mice of the C3H-A^{vy} and C3H-A^{vy}fB strains has been attributed to the use of shavings of eastern red cedar wood in bedding material with the aim of controlling ectoparasites (*Cited in F.C.T.* 1976, **14,** 65). The findings of this latest paper indicate that, in the C3H-A^{vy}fB strain at least, the use of cedar-wood bedding material did not contribute to the high cancer rate.

Mating and breeding of mice of the C3H-A^{vy}fB strain were carried out on bedding of mixed-pine sawdust or on bedding of pine to which red cedar shavings had been added. Females of both groups were maintained after the end of breeding on the same type of bedding as before and examined regularly for mammary tumours. Among the females on mixed pine bedding, mammary tumours occurred in 87% and were detectable after an average of 16 months (range 12–22 months). In those on cedar bedding the incidence was 82%, the tumours being detectable after an average of 17 months (range 10–22 months). The differences between the two groups were not statistically significant. Both groups developed type A and type B adenocarcinomas and adenoacanthomas. At death, all the animals on either type of bedding had hepatomas, which were almost always multiple.

In attempts to identify other possible reasons for the high tumour incidence in these mice, testing of the diet for oestrogenic activity gave essentially negative results and no other significant environmental factor was demonstrated.

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LETTERS TO THE EDITOR

A NEW APPROACH TO INVESTIGATING THE GENETIC TOXICITY OF PROCESSED FOODS

Sir,—Growing dissatisfaction with the standard whole-animal toxicological tests, on the grounds of insensitivity and expense, has led to the development of a variety of short-term test systems for mutagenicity and carcinogenicity (see, for example, Kilbey *et al. Handbook of Mutagenicity Test Procedures*, Elsevier Scientific Publishing Co., Amsterdam, 1977). These tests have been developed for use with pure chemical substances and often involve the treatment of cultured organisms such as bacteria or mammalian cells. In most cases, therefore, it is a problem to utilize these systems for detecting mutagenic activity in foodstuffs because of the latter's physical nature and composition. The problem is greater if the nature and properties of the mutagens that may be present are unknown. The treatment of food with ionizing radiation, the process of interest to the International Food Irradiation Project, gives rise to complex chemical changes which are dependent on food composition and irradiation conditions (see, for example, Elias & Cohen (Editors), *Radiation Chemistry of Major Food Components*, Elsevier Scientific Publishing Co., Amsterdam, 1977). In this case it is clearly most practical to test the foodstuffs *per se*. We have concentrated, therefore, on developing a method for reducing food to a form that can be tested directly in *in vitro* systems and have chosen cultured mammalian cells as our first test organisms.

We have attempted to develop a technique that parallels, as closely as possible, the normal process of digestion, the guiding principles of this endeavour being as follows:

(1) Since the properties of possible mutagens in the food are unknown, nothing should be lost during preparation. Extraction methods are always selective and for this reason have not been used. Although compounds may be destroyed during digestion and lose mutagenic activity, this will probably also occur *in vivo*, if the conditions *in vitro* are comparable.

(2) It is possible that alteration of macromolecules during irradiation could result in the release of active species only on the breakdown of the macromolecule during digestion.

(3) The test material should be diluted as little as possible, either with water or added materials, to effect maximum exposure of the test organisms.

A wide variety of foodstuffs was obtained untreated and then γ-irradiated at suitable doses. Control samples were not irradiated. Where appropriate, the food was cooked in a pressure cooker until judged to be edible. Each food sample was subjected to a digestion procedure decided upon after considerable experimentation, the efficacy of each variation being judged by measuring the production of soluble matter of low molecular weight (dry weight of ultrafiltrate) and estimating glucose, maltose, amino acids, glycerol and fatty acids by standard methods. The food was homogenized in distilled water to give a concentration of 50–200 mg/ml dry weight and the homogenate was acidified to pH 2·0 with 10 N-hydrochloric acid. To each 100 ml of homogenate, 300 mg of three-times crystallized, lyophilized swine pepsin (2500 Anson units/mg) was added, and after incubation for 4 hr with shaking at 37°C, the pH was adjusted to 7·5 with 5 N-NaOH, and 1 g crystalline swine pancreatin and 50 mg sodium taurocholate were added. Incubation was continued for a further 6 hr. During both incubation periods, the pH was monitored continuously and adjusted when necessary. Only general precautions (e.g. the use of sterile glassware solutions and materials) were taken to prevent further contamination of the digest by micro-organisms, particularly in view of the use of low-pH pepsin digestion as the initial step. This point will be investigated more thoroughly in the definitive experiments to be reported later. After digestion, the mixture was centrifuged at 3000 *g* and the supernatant was filtered through a Pellicon ultrafiltration membrane with a nominal molecular weight cut-off of 1000. The osmotic pressure of the ultrafiltrate was measured using the depression of the freezing point of water.

The cell line used to test the ultrafiltrates was the CHO-K_1-BH_4 Chinese hamster ovary, line of Hsie (O'Neill *et al. Mutation Res.* 1977, **45,** 91). Following treatment, measurements of cell survival, mutation frequency and chromosomal aberrations were made according to published protocols (Hsu *et al. ibid* 1977, **45,** 233; O'Neill *et al. loc cit.*; Stetka & Wolff, *Mutation Res.* 1976, **41,** 343). In some experiments, an S9 rat-liver fraction (Ames *et al. Proc. natn. Acad. Sci. U.S.A.* 1973, **70,** 2281) was added to the cultures during treatment to simulate liver metabolism.

With some foodstuffs, such as green vegetables and fruits, digestion had little effect on the amount of material of low molecular weight, but in the case of fish, chicken, potatoes and other protein- or carbohydrate-rich foods, the effect was very marked. In most cases, about 80% of solid material passed the ultrafilter after digestion.

When the osmotic pressure of the ultrafiltrates was adjusted to be close to that of normal culture media (Δ°C approximately 0·7), the digests were found to have no adverse effects on cells incubated

in them for 2–4 hr. The ultimate aim of our work, however, was to obtain cell growth in the digests, so that exposure would be maximal and the products of digestion would be incorporated into the cells in large quantities. To achieve this, it was found necessary to supplement the digests, a process that necessarily diluted the contribution of the food to the medium. This supplementation was minimized by combining different types of food, such as fish plus potato, to give a more balanced product. In this way we have obtained digests that required only the addition of some salts to give very extensive cell growth, comparable to that obtained with standard culture media.

Cells treated in these ways have been used successfully for mutation and chromosome studies and the results of these tests will be reported later. The methods are still at an early stage of development but the results have been sufficiently encouraging to suggest that this approach may be useful not only in our own field of food irradiation but also in the study of other food-processing methods. In addition, the method might be a useful adjunct to the study of the effects of food additives and contaminants where an indirect effect through reaction with food components is possible, or where digestion might affect the activity of the chemical.

The full details of this work, with the results of the tests on various sub-mammalian systems, will be published later.

B. J. Phillips and P. S. Elias,
International Food Irradiation Project,
Postfach 3640, D-7500 Karlsruhe,
Bundesrepublik Deutschland

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CARCINOGENIC RISK ESTIMATION FOR CHLOROFORM: AN ALTERNATIVE TO EPA'S PROCEDURES

Sir.—The Environmental Protection Agency (EPA) has proposed an amendment to the National Interim Primary Drinking Water Regulations as part of the Safe Drinking Water Act. The amendment proposed by EPA would establish a Maximum Contaminant Level for chloroform (and other trihalomethanes) of 0·1 ppm. The Agency indicates in its proposal that this action is necessary because it is felt that the concentrations of chloroform known to exist in drinking-water constitute an appreciable cancer risk to man (*Federal Register* 1978, **43,** 5755).

The magnitude of the cancer risk from chloroform has been estimated by the EPA from data generated at the National Cancer Institute (Report on the Carcinogenesis Bioassay of Chloroform, Bethesda, MD, 1976). In this study, long-term administration of chloroform at very high doses was found to produce tumours in laboratory rodents. However, there is reason to believe that the risk extrapolation performed by EPA seriously overestimates the actual potential of chloroform to induce cancer in man. There are two major reasons why this particular risk estimate is probably inaccurate. First, the EPA has failed to consider important data concerning the mechanisms through which chloroform exerts its toxicity. This has led to a major error in making species/species extrapolations of carcinogenic risk. Secondly, the NCI study was carried out with very high doses of chloroform. At slightly lower doses, the relative carcinogenicity of chloroform falls sharply. This suggests that there may be detoxification mechanisms which effectively protect the animals until they are overwhelmed by very large doses. Detailed discussion of these two points follows. In view of the enormous economic impact of regulations such as these, it is imperative that the risk estimation be performed as accurately as possible so that the risk/benefit ratio can be properly considered.

Species/species extrapolation for chloroform toxicity

The Carcinogen Assessment Group of EPA performs risk extrapolations under the assumption that the carcinogenic process does not have a threshold. Since it is impossible to prove or disprove this point experimentally, further argument is futile and we will proceed without resolving this question. The risk extrapolation from animals to man involves two steps.

(A) First, the excess cancer incidence (if statistically different from the control) is empirically fitted to a mathematical model relating dose and response. The most commonly used models are:

$$\text{(i) Linear } P = \beta d$$
$$\text{(ii) `One hit' } P = 1 - e^{-\beta d}$$

where P is the fractional incidence of excess tumours, d is the average daily dose in mg/kg, and β is a dimensionless parameter to be estimated from the experimental data.

(B) Next a species correction factor is applied to β. This has been chosen to be the cube root of the ratio of the body weights (i.e. adjustment on a dose/unit surface area). Assuming an average weight of 30 g for mice, 500 g for rats and 70 kg for man, this translates as a 13·3-fold increase for a mouse-to-man extrapolation and a 5·19-fold increase for a rat-to-man extrapolation. Finally the risk to man is calculated by substituting the human dose into the equation. The net result is that man is always considered to be more sensitive to a carcinogen than the small animal species.

This type of species/species extrapolation was developed by Rall (*Envir. Res.* 1960, **2,** 360) for application to a group of 18 antineoplastic agents. These chemicals produce *direct* cytotoxicity and, according to the author "... are generally not involved in the variable drug metabolizing systems ...".

In the case of chloroform, however, metabolism plays a different role in toxicity. This is because the toxicity of chloroform is apparently not due to the parent molecule itself, but rather to the production of reactive metabolite(s) which bind covalently to tissue proteins (Brodie *et al. Proc. natn. Acad. Sci.* 1971, **68,** 160; Ilett *et al. Expl mol. Path.* 1973, **19,** 215; Reid, *Proc. Fifth Int. Congr. Pharmac., San Francisco, CA,* 1973; Reid & Krishna, *Expl mol. Path.* 1973, **18,** 80; Reid *et al. Am. Rev. resp. Dis.* 1973, **107,** 539). Chloroform thus appears to belong to a class of chemicals that require metabolic activation for toxicity. Consequently, any species variation in the capacity to metabolize chloroform should dramatically affect the oncogenicity of this compound.

In order to assess the relative importance of these factors, we have attempted to predict the carcinogenicity of chloroform to rats on the basis of the tumour incidence observed in mice (NCI, *loc. cit.*). Since rats were also tested in the NCI study of chloroform the observed incidence of rat tumours can serve to indicate the validity of the predictions.

First, we used the interspecies extrapolation procedure of the EPA (procedure A). The rats in the NCI study had a mean body weight of approximately 500 g, while the mice had a mean body weight of approximately 30 g. The cube root of the body-weight ratio (500/30) is 2·55. The rats should be *more* sensitive to chloroform by this factor. That is, a dose of chloroform that produced 10% tumours in a group of mice should produce 25·5% tumours in a group of rats.

Secondly, we have attempted to predict carcinogenicity from the relative rates of production of the toxic chloroform metabolite (procedure B). Several investigators have studied the metabolism of chloroform in rats and mice and concluded that mice metabolize chloroform more rapidly than do rats. The most complete studies are those of Brown *et al.* (*Xenobiotica* 1974, **4,** 151), who adminis-

tered a 60-mg/kg dose of chloroform orally to rats, squirrel monkeys and three strains of mice. They recovered very little unmetabolized chloroform from the mice (Table 1). In the rat, three times as much unmetabolized chloroform was recovered, while in the monkey, 13 times as much unaltered chloroform was exhaled. Fry *et al.* (*Archs int. Pharmacodyn. Thér.* 1972, **196,** 98) administered chloroform to human volunteers and found that 17–66% of the material was exhaled unchanged. However, the human data may be somewhat misleading. The dose of chloroform used in man (7 mg/kg) was much lower than that given to the animals. Consequently, the relative amount of chloroform that would be metabolized at higher doses may have been overestimated.

Table 1. *Interspecies comparisons: chloroform*

Species	Oral dose (mg/kg)	$CHCl_3$ excreted unchanged (% of dose)
Mouse	60	6*
Rat	60	20*
Monkey	60	78*
Man	*c.*7	17–66†

*Brown *et al. Xenobiotica* 1974, **4,** 151.
†Fry *et al. Archs int. Pharmacodyn. Thér.* 1972, **196,** 98.

Although it is obviously difficult to make quantitative predictions from this type of metabolic data, it is clear that procedure B (relative metabolic capacity) leads to the opposite prediction from that given by the EPA procedure (A). Since the mouse metabolizes chloroform most rapidly, and presumably produces more of the toxic metabolite, it should be more sensitive to chloroform than the rat. Therefore, the interspecies correction factor is used in reverse in this case.

The results of these predictions may be summarized as follows:

(1) Predicted from EPA's surface area model (Rall, *loc. cit.*)	Rat > Mouse (2·55-fold)
(2) Predicted by metabolic rates (Brown *et al. loc. cit.*)	Mouse > Rat (2·55-fold)
(3) Observed in NCI bioassay (NCI, *loc. cit.*)	Mouse > Rat (4·2-fold)

For the purpose of evaluating the actual carcinogenicity in the two species, the β parameter is estimated for the most sensitive sex in the NCI bioassay according to the one hit model. The relative sensitivity is then approximated for low doses as the ratio of the two β values. Clearly procedure B is a better predictor than procedure A, for the actual tumour incidence is 4·2-fold higher in the mouse at equivalent doses of chloroform. Thus the application of the EPA technique for interspecies extrapolation has produced an error of 10·7-fold in estimating the sensitivity of the rat from the mouse data.

It is also noteworthy that extrapolation of these data to man with these two procedures gives very different results. The EPA procedure (procedure A) produces human risk estimates that are inconsistent. The mouse data predict a human risk 11-fold higher than the rat-data prediction. In contrast, with procedure B, the two risk estimations agree very well. Risk predicted from the mouse data is only 1·6-fold higher than risk predicted from the rat data.

Based on these considerations we would consider man to be the least sensitive of the three species to the carcinogenic action of chloroform. This estimate is based on the data of Fry *et al.* (*loc. cit.*) as well as on the general principle that man normally metabolizes materials much more slowly than the small laboratory animals (Weiss *et al. Int. J. clin. Pharmac.* 1977, **15,** 572). Although the absolute magnitude of the risk is dependent upon the particular mathematical model employed in step A, incorrect application of the 'species correction factor' causes an increase of more than two orders of magnitude in the estimated risk to man. Instead of an excess cancer risk "...on the order of 10^{-4}–10^{-5}...." (EPA, Statement of the Basis and Purpose for an Amendment to the National Interim Primary Drinking Water Standards on Trihalomethanes: Office of Water Supply Criteria and Standards Division, Washington, DC, 1978), we feel that the risk cannot be higher than about 1% of this figure.

In addition to chloroform, there are many other instances where the relative carcinogenicity of chemicals correlates well with their rate of activation to a reactive species. For instance, vinylidene chloride (VDC) is metabolized more extensively in the mouse than in the rat (McKenna *et al. Envir. Hlth Perspect.* 1977, **21,** 99). Tumours have been observed in mice exposed to VDC (Maltoni, *ibid* 1977, **21,** 1) while studies in rats have failed to reveal a discernible oncogenic response (Maltoni, *loc. cit.*; Rampy *et al. Envir. Hlth Perspect.* 1977, **21,** 33; Viola & Caputo, Cancerogenicity Studies on Vinylidene Chloride: paper presented at NIEHS Conference on Comparative Metabolism and

Toxicity of Vinyl Chloride Related Compounds, Bethesda, MD, 2–4 May 1977). Similarly 2-acetaminofluorene is metabolized to a reactive electrophile and the carcinogenicity of this material correlates well with the relative abundance of enzymes to carry out this activation in various species (Miller, Report to Conference on Environmental Carcinogenesis, Michigan State University, East Lansing, MI, 22–23 May 1978). Trichloroethylene is carcinogenic in B6C3F1 mice but not in Osborne–Mendel rats (NCI, Report on the Carcinogenesis Bioassay of Trichloroethylene, Bethesda, MD, 1976). This correlates well with the greater capacity of mouse microsomes to catalyse incorporation of trichloroethylene metabolites into DNA (Banerjee & Van Duuren, *Cancer Res.* 1978, **38**, 776).

High dose/low dose extrapolations

For most risk estimations it is customary to extrapolate from high doses, where experimentally observable rates of tumour induction exist, to doses in the environment several orders of magnitude lower. For instance, the doses of chloroform used in the NCI study (Report on the Carcinogenesis Bioassay of Chloroform, Bethesda, MD, 1976) are about 100,000 fold higher than the Maximum Contaminant Level proposed by the EPA. This raises important questions about the validity of such extrapolations.

There are many instances where toxicity (and presumably also carcinogenicity) increases disproportionately once the normal detoxification mechanisms are overwhelmed. An example of this type of behaviour is the work recently reported by McKenna *et al.* (*loc. cit.*) for VDC. It has been postulated that VDC is metabolized in the body to a reactive electrophile which reacts with either endogenous glutathione (detoxification) or various macromolecules (toxic action). In the rat, glutathione is depleted by exposure to high levels of VDC (Jaeger *et al. Expl mol. Path.* 1974, **20,** 187). Furthermore, fasting the rats prior to VDC exposure further depleted the glutathione and dramatically increased the hepatotoxicity of VDC. More importantly, McKenna *et al.* (*loc. cit.*) noted a sudden disproportionate increase in macromolecular binding of VDC metabolites as glutathione was depleted by more than 30%. These observations are particularly important in view of the fact that chloroform is also known to deplete glutathione, and that chloroform toxicity can be antagonized by administration of cysteine, a glutathione precursor (Docks & Krishna, *Expl mol. Path.* 1976, **24,** 13).

The validity of using high doses of chloroform to predict the effects of low doses may be tested by examining the data of the NCI (Report on the Carcinogenesis Bioassay of Chloroform, Bethesda, MD, 1976) and of Roe (Preliminary Report of Long Term Tests of Chloroform in Rats, Mice and Dogs, personal communication, 1976). Data from the highest dose group will be used to estimate the parameters for the one hit model and the linear model. Tumour incidence for lower doses will then be predicted and compared to observed incidence rates. Because of the different spontaneous tumour rates for the various studies, the most logical way to compare results from the studies is through excess tumours calculated from Abbott's correction:

$$P_{excess} = \frac{P_{treatment} - P_{control}}{1 - P_{control}}$$

Thus our models will be:

$$P_{excess} = \beta x \text{ (linear model)}$$
$$P_{excess} = 1 - e^{-\beta x} \text{ (one hit model)}$$

Upper 95% confidence limits are also calculated for the observed number of tumours. These are exact confidence intervals when there is no spontaneous background rate (i.e. when Abbott's correction is not necessary) or when the observed frequency rate in the treated group is zero. For non-zero frequencies with non-zero spontaneous rates the confidence interval is calculated using the normal approximation to the binomial distribution and using the appropriate standard deviation for a ratio from the Taylor series expansion of the ratio.

The predictions, as well as the actually observed incidences, are listed in Table 2. Neither the linear model nor the one hit model adequately predict the tumour incidence below 200 mg chloroform/kg. In most cases, even the upper 95% confidence interval for the observed tumour rates is below the predicted values. For instance, the NCI male mice given 138 mg/kg would be predicted to have 2·4 times more tumours than were actually observed. The tumour incidences observed by Roe (*loc. cit.*) in mice exposed to 60 mg/kg or less are even further from the predicted values (Table 2). Since Roe utilized four different strains of mice, and none of these results are well described by the model, the failure of the extrapolation is probably due to a deficiency in the model rather than to strain differences. However the only definitive procedure to establish the dose-dependency of the carcinogenic effect of chloroform is for the NCI to repeat the carcinogen bioassay over a wider range of doses. Until this has been done, there will be a great deal of uncertainty about the lower end of the dose-response curve.

Experience with human exposure to chloroform also appears to be inconsistent with EPA's risk assessment. For example, a study of British workers in a confectionery factory indicated chronic exposure (up to 10 years) to concentrations of 50–125 ppm chloroform vapours (Challen *et al. Br. J. ind. Med.* 1958, **15,** 243). If we assume that the two routes of exposure (oral and inhalation)

Table 2. *Predicted and observed incidences of excess liver tumours in chloroform-treated mice*

Strain	Dose (mg/kg)	Predicted tumours (%)		Observed tumours	
		Linear*	One hit†	Incidence (%)	Upper 95% confidence interval
		Males			
B6C3F1	277	—	—	92	—
	138	46	72	30	47
C57Bl	60	20	42	0	6
CBA	60	20	42	0	2
CF/1	60	20	42	2 (NS)	13
ICI	60	20	42	7 (NS)‡	18
	17	6	14	8 (NS)	20
		Females			
B6C3F1	477	—	—	95	—
	238	47	78	80	91
ICI	60	12	31	0	8
	17	3	10	0	8

NS = Observed tumour incidence not significantly different from control incidence
* $P = 1 - e^{-(0.00912 \times \text{dose})}$ for males and $1 - e^{-(0.00628 \times \text{dose})}$ for females.
†$P = 0.00332 \times$ dose for males and $0.00199 \times$ dose for females.
‡ A 22% incidence of excess *kidney* tumours seen in this strain was significantly different from that in the controls.

are essentially equivalent, these workers would be predicted to have a lifetime cancer risk of 20–40% in the group exposed for a mean of 5·4 years (EPA one hit model, NCI female mouse data with surface area correction). These exposures were documented 20 years ago, so that even with a long latency period, increased cancer incidence should be demonstrable if the EPA risk estimation is valid. Similar reports exist from the chemical industry (Bomski *et al. Arch. Gewerbepath. Gewerbehyg.* 1967, **24,** 127; Lehman & Schmidt-Kehl, *Arch. Hyg.* 1936, **116,** 131). However, to date, no epidemiology study convincingly links industrial exposure to chloroform with an increased cancer risk.

In summary, there are several reasons for believing that the current EPA risk estimation for chloroform is seriously in error. We feel that definitive studies of the metabolism of chloroform in various species must be carried out in order to allow a rational species/species extrapolation to be performed. Furthermore, there is evidence of a sudden change in the shape of the chloroform dose-response curve below 200 mg/kg. A complete experiment to evaluate the carcinogenicity of chloroform at lower doses must be carried out before high dose/low dose extrapolations can be performed. A study of the pharmacokinetics of chloroform in the selected species would be valuable in selecting appropriate doses for this study.

There is good evidence that there are two classes of carcinogens. One class appears to produce its effect directly (e.g. β-propiolactone) and for these agents, at least when given orally, it may be appropriate to apply surface-area correction factors (procedure A). Compounds in the second class require metabolic activation before they become carcinogenic (e.g. 2-acetylaminofluorene). In this case, the relative rate of metabolic activation appears to be the most reliable means of making interspecies extrapolation of carcinogenic risk. Consequently, we strongly suggest that the NCI develop an appropriate technique for distinguishing between these two classes of carcinogens and that EPA then apply appropriate species/species extrapolation techniques before proposing any regulations based on risk assessment.

R. H. Reitz and P. J. Gehring,
Toxicology Research Laboratory,
1803 Building, Dow Chemical Company,
and
C. N. Park,
C.R. Mathematical Applications Group,
1707 Building, Dow Chemical Company,
Midland, MI 48640, USA

MEETING ANNOUNCEMENTS

FACTORS IN CARCINOGENESIS

The Second Conference on Inorganic and Nutritional Aspects of Cancer, organized by the International Association of Bioinorganic Scientists, will be held at La Jolla, California, on Tuesday, 2 January to Friday, 5 January 1979. The topics to be discussed will include metal carcinogenesis, cancer epidemiology, theoretical aspects of carcinogenesis, occupational cancers, trace minerals and cancer prevention.

Further information and application forms may be obtained from Dr. G. N. Schrauzer, IABS Cancer Conference, Department of Chemistry, University of California, San Diego Revelle College, La Jolla, CA 92037, USA (telephone no. (714) 452–4463).

CONSULTANT'S COURSE AT UNIVERSITY OF CALIFORNIA HOSPITAL

The Seventh Annual Consultant's Course will be held at the University of California Medical Center, San Francisco, on Tuesday, 6 March through Friday, 9 March, terminating on 10 March with the semi-annual meeting of the San Francisco Dermatologic Society at the Medical Center. The faculty largely consists of members of the Department of Dermatology. Major guest lectureships include the Frank Allende and Ingram lectureships. Further information may be obtained from: Extended Programs in Medical Education, University of California, San Francisco, CA 94143, USA (telephone no. (415) 666–4251).

FORTHCOMING PAPERS

It is hoped to publish the following research papers in the next issue of *Food and Cosmetics Toxicology*:

Short-term toxicity study of sorbitan monolaurate (Span 20) in rats. By B. R. Cater, K. R. Butterworth, I. F. Gaunt, J. Hooson, P. Grasso and S. D. Gangolli.

Long-term toxicity study of sorbitan monostearate (Span 60) in mice. By R. J. Hendy, K. R. Butterworth, I. F. Gaunt, I. S. Kiss and P. Grasso.

Short-term toxicity study of sorbitan mono-oleate (Span 80) in rats. By A. J. Ingram, K. R. Butterworth, I. F. Gaunt, P. Grasso and S. D. Gangolli.

Transfer of polychlorinated biphenyls from mothers to foetuses and infants. By Y. Masuda, R. Kagawa, H. Kuroki, M. Kuratsune, T. Yoshimura, I. Taki, M. Kusuda, F. Yamashita and M. Hayashi.

Teratogenicity of acrylonitrile given to rats by gavage or by inhalation. By F. J. Murray, B. A. Schwetz, K. D. Nitschke, J. A. John, J. M. Norris and P. J. Gehring.

Disposition of zinc pyrithione in the rat. By J. H. Wedig, R. A. Wentworth, M. A. Gallo, J. G. Babish and J. D. Henion.

Effect of nitrilotriacetate (NTA) on cation balance in the rat. By R. L. Anderson and R. L. Kanerva.

Hypercalcinuria and crystalluria during ingestion of dietary nitrilotriacetate. By R. L. Anderson and R. L. Kanerva.

Inflammation expérimentale et taux protéique alimentaire chez des rats intoxiqués subchroniquement au nabame. Par A. Périquet et R. Derache.

The effect of L-tryptophan and certain other amino acids on liver nitrosodimethylamine demethylase activity. By R. P. Evarts and M. H. Mostafa. (Short Paper)

A synergistic effect of nitrosodimethylamine on sterigmatocystin carcinogenesis in rats. By K. Terao, T. Aikawa and K. Kera. (Short Paper)

Reorganization of the UK total diet study for monitoring minor constituents of food. By D. H. Buss and D. G. Lindsay. (Short Paper)

T-2 toxin mycotoxicosis in the guinea-pig. By D. B. DeNicola, A. H. Rebar, W. W. Carlton and B. Yagen. (Short Paper)

Induction of duodenal serotonin production by dietary sodium selenite and aflatoxin B_1. By J. H. Lalor, T. D. Kimbrough and G. C. Llewellyn. (Short Paper)

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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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